Ironing out the Pathophysiology of Neurodegeneration with Brain Iron Accumulation (NBIA)

*Clinical Investigations and Disease Modelling Yield Novel Evidence of Systemic Dysfunction and Provide a Robust and Accurate Disease Model of NBIA*

by

Michael Minkley
BSc, University of Victoria, 2013

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the Department of Biology

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Abstract

Neurodegeneration with Brain Iron Accumulation (NBIA) disorders, such as Phospholipase A2G6-Associated Neurodegeneration (PLAN) and Pantothenate Kinase-Associated Neurodegeneration (PKAN), are a group of rare early-onset, genetic disorders characterized by neurodegeneration and iron accumulation inside of the basal ganglia (BG), which is accompanied by progressive motor symptoms. In order to address the limitations in available models of NBIA, a B6.C3-Pla2g6m1J/CxRwb mouse model of PLAN was characterized. This model demonstrated key hallmarks of the disease presentation in NBIA, including a severe and early-onset motor deficit, neurodegeneration inside of the substantia nigra (SN) including a loss of dopaminergic function and the formation of abnormal spheroid inclusions as well as iron accumulation. The capture of these hallmarks of NBIA makes this an ideal animal research model for NBIA.

Additionally, exploration of candidate systemic biomarkers of NBIA was performed in a case study of a patient with PLAN and in a cohort of 30 patients with PKAN. These investigations demonstrated reductions in transfer and slight, but not significant elevations in soluble transferrin receptor. No significant difference was seen in serum iron parameters. A systemic disease burden including chronic oxidative stress; elevated malondialdehyde, and inflammation; elevated C-reactive protein (CRP), IL-6 and TNFα was noted in both investigations. A number of candidate protein biomarkers including: fibrinogen, transthyretin, zinc alpha-2 glycoprotein and retinol binding protein were also identified. These markers correlated with measures of the severity of iron loading in the globus pallidus (GP); based on R2* magnetic resonance imaging (MRI) and the severity of motor symptoms (Barry-Albright Dystonia Rating Scale) making them potential candidates markers of dysfunction in NBIA. In the patient with PLAN, 37 weeks of therapy with the iron chelator deferiprone (DFP) as well as 20 months of therapy with the antioxidants alpha lipoic acid (ALA) and n-acetylcysteine (NAC) were efficacious in reducing the systemic oxidative and inflammatory disease burden, but it did not significantly alter the progression of the disease. In the antioxidant therapy, this efficacy was primarily due to ALA. When the cohort of patients with PKAN were treated with DFP
for 18 months it was highly efficacious in lowering brain iron accumulation in the GP. No significant reduction in the speed of disease progression was seen in DFP treated patients compared to placebo based on initial analysis. Similar to the PLAN patient, DFP also mitigated the systemic disease burden in PKAN patients. In both cases DFP was well tolerated and had minimal impact on serum iron levels, TIBC and transferrin saturation. Collectively these investigations provide valuable insights into disease progression in NBIA. They also provide tools to aid further investigations in NBIA. These are provided in the form of a well-characterized B6.C3-Pla2g6<sup>m1J/CxRwb</sup> model of PLAN, which robustly captures the disease presentation seen in patients, as well as a panel of systemic blood-based markers of disease burden in NBIA and candidate markers of dysfunction in NBIA. These markers were used to assess two novel therapies in NBIA chelation with DFP and antioxidant therapy with ALA and NAC.
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List of Abbreviations

ALA – Alpha-lipoic acid
ALT – Alanine aminotransferase
aNAD – Atypical neuroaxonal dystrophy
BAD – Barry-Albright Dystonia Scale
BBB – Blood-brain barrier
BECs – Brain endothelial cells
BG – Basal ganglia
BHT – Butylated hydroxy toluene
BUN – Blood urea nitrogen
CNS – Central nervous system
CoA – Co-enzyme A
CRP – C-reactive protein
DAB – Diaminobenzidine
DFP – Deferiprone
DMT1 – Divalent metal transporter 1
DN - Dopaminergic
FP - Ferroportin
GABA – Gamma aminobutyric acid
GDS – Global deterioration score
GP – Globus pallidus
GPe – Globus pallidus external segment
GPi – Globus pallidus internal segment
HIF – Hypoxia inducible factor
HPLC – High performance liquid chromatography
HSS - Halloverden-Spatz syndrome
IAP – Intracisternal A particle
IL-10 – Interleukin 10
IL-1β – Interleukin 1 beta
IL-6 – Interleukin 6
INAD – Infantile neuroaxonal dystrophy
iPLA2β – Phospholipase A2G6
IRE – Iron response element
IRP – Iron response element binding protein
ISC – Iron-sulphur cluster
LC – Liquid chromatography
LC-MS – Liquid chromatography-mass spectrometry
LDH – Lactate dehydrogenase
LIP – Labile iron pool
LPI – Labile plasma iron
MALDI-TOF - Matrix-assisted laser desorption ionization time-of-flight
MDA - Malondialdehyde
MRI – Magnetic resonance imaging
MRM – Multiple reaction monitoring
MS – Mass spectrometry
NAC – N-acetyl cysteine
NBIA – Neurodegeneration with Brain Iron Accumulation
NTBI – Non-transferrin bound iron
PANK2 – Pantothenate Kinase 2
PARK14 – Parkinson’s disease 14 (PLA2G6 associated form)
PBS – Phosphate-buffered saline
PD – Parkinson’s disease
PedsQL – Pediatric quality of life inventory
PET – Positron emission tomography
PFA - Paraformaldehyde
PKAN – Pantothenate kinase-associated neurodegeneration
PLAN – Phospholipase A2G6-associated neurodegeneration
PNS – Peripheral nervous system
RBP – Retinol binding protein
ROS – Reactive oxygen species
SN – Substantia nigra
SNc – Substantia nigra pars compacta
SNr – Substantia nigra pars reticulata
Tf - Transferrin
TfR – Transferrin receptor
TfR1 – Transferrin receptor 1
TfR2 – Transferrin receptor 2
TH – Tyrosine hydroxylase
THY1 – Thymus cell antigen 1
TIBC – Total iron binding capacity
TNFa – Tumour necrosis factor alpha
TTH - Transthyretin
UPDRS – Unified Parkinson’s disease rating scale
YFP – Yellow fluorescent protein
ZAG – zinc alpha-2 glycoprotein
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Chapter 1. Phospholipase A2G6-Associated Neurodegeneration and Pantothenate Kinase-Associated Neurodegeneration

Recent advances in genetic sequencing and brain imaging techniques have enabled better delineation of a novel group of rare early-onset, familial, monogenic neurodegenerative disorders that primarily target the basal ganglia (BG). These disorders are characterized by extrapyramidal symptoms as well as cognitive and motor dysfunction. Collectively, these disorders are known as Neurodegeneration with Brain Iron Accumulation (NBIA). Previously all forms of NBIA were classified under Halloverden-Spatz syndrome (HSS), but genetic methods of investigation have revealed this to be a heterogenous group of disorders. As their name would suggest, one of the predominant hallmarks of these disorders is iron accumulation inside of the dysfunctional BG (Hogarth 2015). Iron accumulation inside the brain is a well-documented process, which typically only occurs during the aging process (Hardy et al., 2005; Hill & Switzer, 1984). The modest increase that occurs during aging is not typically pathogenic; indicative of a robust ability to handle iron within the brain. However, disruptions of this process leading to excess and unregulated iron accumulation within the brain is a hallmark of a number of neurodegenerative disorders including Alzheimer’s disease and Parkinson’s disease (PD) (Rouault 2013) as well as the highly abnormal early-onset and rapid iron accumulation seen in NBIA. Another common hallmark of NBIA disorders is the formation of abnormal inclusion-like spheroids within degenerating areas of the central nervous system (CNS), such as the BG. No universal description of these spheroids has been provided in NBIA, but they have been noted to form in association with abnormal, dysfunction mitochondrial in the degenerating axons of neurons in some studies. Parallels can be drawn to the formation of Lewy body inclusions in PD, but further research efforts are needed to characterize the prevalence and nature of these spheroids in NBIA in order to verify if the accumulation of ubiquinated proteins and alpha-synuclein is a hallmark of these spheroids as it is in PD.

Though they are relatively rare; with incidences in the range of 1 in 1,000,000 (Hogarth, 2015), NBIA disorders have many parallels to more common neurodegenerative
disorders, such as PD. Due to these parallels, research efforts in NBIA help to provide insight into two key shared hallmarks: neurodegeneration and abnormal iron accumulation inside nuclei of the BG, such as the substantia nigra (SN) and globus pallidus (GP). For further details of the layout, architecture and function of the BG see Chapter 2 as well as the reviews by Calabresi, Picconi, Tozzi, Ghiglieri, & Di Filippo (2014), Alexander & Crutcher, (1990) and Parent & Hazrati, (1995), which provide details of the location and circuitry of the BG. Furthermore, the well characterized genetic background of patients with NBIA disorders contrasts with the heterogenous background that is seen in PD. Variability within the patient population can obscure the significance of findings. This makes NBIA disorders ideal models for investigating these hallmarks. Research into the underlying mechanisms which lead to dysfunction in NBIA not only benefits the relatively small group of individuals affected by these disorders, but also the larger patient population suffering from disorders featuring neurodegeneration of the BG.

To date, 10 different NBIA disorders have been genetically characterized. A table fully summarizing each of the 10 characterized disorders is presented in Appendix A. Broadly these can be split into two groups: those with mutations in proteins directly related to iron metabolism and those with mutations in other proteins, such as fatty acid metabolism (Levi & Finazzi, 2014). A series of thorough reviews on NBIA are available which cover these disorders in detail (Gregory, Polster, & Hayflick, 2009; Gregory & Hayflick, 2013; Hogarth, 2015; Levi & Finazzi, 2014; Schneider, Hardy, & Bhatia, 2012). In addition to the characterized forms of NBIA, a number of idiopathic cases of NBIA still exist making it likely that the number of NBIA disorders will continue to rise as novel causative genes are discovered. Currently, the two most common NBIA disorders are Pantothenate Kinase-Associated Neurodegeneration (PKAN) and Phospholipase A2G6-Associated Neurodegeneration (PLAN) (Hogarth, 2015), Improved understanding of the mechanisms pathophysiology these two disorders, which make up the majority of NBIA cases, is the primary focus of this work. Secondary to this, findings will also be discussed in the context of other disorders such as PD.
1.1. Phospholipase A2G6 Associated Neurodegeneration (PLAN)

1.1.1. Molecular and Genetic Basis of PLAN

Phospholipase A2G6 associated neurodegeneration (PLAN) is an NBIA disorder that was genetically characterized in the work of Morgan et al. (2006). Morgan et al. (2006) determined that PLAN represents a subtype of NBIA, unique from PKAN, which is caused by mutations in the \( \text{PLA2G6} \) gene encoding the calcium independent phospholipase A2G6 (iPLA2\( \beta \)). iPLA2\( \beta \) is an A2 type phospholipase, which means it is responsible for cleaving the sn-2 acyl bond of phospholipases (See Figure 1-1). Similar to PANK2, iPLA2\( \beta \) appears to be involved in a wide array of cellular functions (Morgan et al., 2006; Schneider, Bhatia, & Hardy, 2009; Strokin, Seburn, Cox, Martens, & Reiser, 2012; Sun et al., 2010). It has proposed roles in membrane dynamics and homeostasis through phospholipid remodeling, as well as leukotriene and prostaglandin synthesis, apoptosis and inflammation (Morgan et al., 2006; Schneider et al., 2009; Sun et al., 2010). Pathogenic mutations found in PLAN generate enzymatically inactive isoforms which inhibit the actions of the phospholipase and render it inactive (Morgan et al., 2006). These defects in iPLA2\( \beta \) have the potential to have wide ranging effects in the cellular environment.

Figure 1-1. Generalized depiction of the enzymatic activity of A2 type phospholipases (PLA2), such as iPLA2\( \beta \). iPLA2\( \beta \) is implicated in the pathogenesis of PLA2G6-Associated Neurodegeneration (PLAN).
1.1.2. Symptoms and Presentation of PLAN

Typical symptoms of PLAN are reviewed below, however heterogeneity in the expression of these symptoms is seen in affected patients. The classical symptomology of PLAN is also known as infantile neuroaxonal dystrophy (INAD). INAD presents early in life, between 6 months and 3 years of age (Gregory & Hayflick, 2013; Hogarth, 2015). The progression of INAD is rapid and devastating, early signs include a slowing and eventual regression of normal development (Gregory & Hayflick, 2013; Hogarth, 2015). This regression is predominantly psychomotor and is often followed by the emergence of hypotonia, optic atrophy and spastic tetraparesis (Gregory et al., 2009; Gregory & Hayflick, 2013; Hogarth, 2015). Notably, a small subset of INAD cases have a later emergence and delayed progression; this subset of INAD cases is termed atypical infantile neuroaxonal dystrophy (aNAD) (Gregory et al., 2009). Minor pathological symptoms seen in INAD are more pronounced in aNAD including gait ataxia, dysarthria and dystonia (Gregory et al., 2009; Hogarth, 2015).

Mutations in iPLA2β have also been demonstrated to be responsible for a progressive dystonia-parkinsonism syndrome which manifests in late adolescence or early adulthood (Hogarth, 2015). Progression is rapid and is accompanied by abnormal eye movements and cognitive decline (Hogarth, 2015). The strong similarity between this presentation of PLAN, which includes dystonia-parkinsonism, iron accumulation inside the SN as well as a loss of dopaminergic function, has led to mutations in iPLA2β to also be classified as a causative gene in PD; PARK14 (Yoshino et al., 2010; Q. Zhou et al., 2016). The dual characterization of the later-onset dystonia-parkinsonism form of PLAN as both NBIA and PD is also supported by the presence of Lewy body and synucleinopathy pathology in cases of PLAN/PARK14 (Miki et al., 2017; Paisán-Ruiz et al., 2012). An example of this was seen in post-mortem examination of the brains of patients with confirmed PLA2G6 mutations by Gregory et al. (2008). This examination showed iron accumulations inside the GP and other regions of the BG, prominent and widespread Lewy bodies, dystrophic neuritis and axonal swelling (Gregory et al., 2008). Additionally, iron accumulation which is present in 50% of INAD patients is found in all patients with
PARK14 (Gregory et al., 2008). Collectively, these shared disease hallmarks provide further evidence for the strong parallels between NBIA and PD.

It is likely that these sub-classifications of PLAN; which show a range of age of onset as well as severity of progression, are related to the nature of the inactivating mutations in iPLA2β found in each patient. A recent study revealed that the nature and location of mutations in the PL2G6 gene may be responsible for the heterogeneity between the various forms of PLAN (Engel, Jing, O’Brien, Sun, & Kotzbauer, 2010). Thus, the severity of impairment of iPLA2β function may be directly related to the presentation of PLAN, with a more severe mutation, such as in the active site of the protein, resulting in the more rapid onset and quicker progression of INAD, which also sees a more widespread pathology and a later onset dystonia-parkinsonism phenotype seen in mutations with less impact on iPLA2β catalytic activity, such as those that occur in regulatory regions (Engel et al., 2010).

1.1.3. Pathophysiology of PLAN

In a large group of patients with confirmed PL2G6 mutations cerebellar atrophy was seen (Gregory et al., 2008). Iron accumulation inside the BG is often, but not universally seen in INAD and aNAD (Gregory et al., 2008). It is possible that this iron accumulation, like the iron accumulation seen in aging requires a longer time to accumulate. This accumulation is predominantly in the GP, but it is also frequently seen in the substantia nigra (SN) on magnetic resonance imaging (MRI) (Gregory & Hayflick, 2013; Hogarth, 2015). Initial iron deposition may be subtle requiring susceptibility weighted imaging (Gregory & Hayflick, 2013; Hogarth, 2015). Iron accumulation is seen with variable severity in the GP and SN of nearly all of the patients with PLAN/PARK14 (Gregory & Hayflick, 2013; Hogarth, 2015). Optic and cerebellar atrophy are also seen in INAD and aNAD (Gregory & Hayflick, 2013; Hogarth, 2015). Dystrophic neuroaxonal spheroids characteristic of INAD are found in the brainstem, peripheral nerves, BG and spinal cord (Hogarth, 2015).
1.1.4. Models and Research Efforts in PLAN

The specific cellular defects caused by a deficiency of iPLA2β are still being explored. It is likely that loss of the phospholipase activity of iPLA2β leads to dysfunctional phospholipid metabolism and remodeling. Evidence for this theory was presented by a recent study performed by Cheon et al. (2012) in a mouse model; which highlighted deficits in phospholipid metabolism: including reduced metabolism of certain fatty acids, such as docosahexaenoic acid as well as alterations in lipid-metabolizing enzyme expression and brain fatty acid content. This was caused by a genetic deficiency of iPLA2β. The presence of neurological symptoms was not noted in these mice, so it is still unclear how these disruptions may lead to neurodegeneration in PLAN.

Cerebellar atrophy was seen in investigations in another knockout mouse model of PLAN (Zhao et al., 2011). The mice were positive for a full genetic ablation of iPLA2β. By 13 months of age, prominent cerebellar atrophy was seen in the mice. This matches the pathophysiology seen in PLAN patients. Cerebellar atrophy was accompanied by microglial activation; evidenced by increased expression of the proinflammatory cytokines IL1β and TNFα (Zhao et al., 2011). This proinflammatory response was demonstrated to precede the onset of cerebellar atrophy (Zhao et al., 2011). This suggests that cerebellar inflammation may be a contributing factor in the neurodegeneration observed in PLAN. However, the onset of symptomology was delayed in these mice and no overt motor symptoms or iron accumulation was noted.

Interestingly, motor symptoms were seen in another induced knockout model of PLAN. The variability between these models highlights the hypothesis of Engel et al. (2010); different mutations in iPLA2β in these mouse models lead to variable disease presentation. Shinzawa et al. (2008) showed that these iPLA2β deficient mice develop motor deficits, beginning with impaired performance on wire hang tests at 30 to 50 weeks of age. Neuropathological investigations in these mice revealed the widespread formation of spheroids and vacuoles in axons in the CNS and peripheral nervous system (PNS) by 2 years of age (Shinzawa et al., 2008). Investigations in younger mice showed
that the size, number and prevalence of these spheroids increased over the course of disease progression (Shinzawa et al., 2008).

Confirmation of the widespread formation of spheroid-like structures due to a iPLA2β deficiency was provided by the work of Beck et al. (2011) who examined the spinal cord and sciatic nerves of mice in a sperate iPLA2β knockout model of PLAN. They noted the formation of tubulovesicular structures which were composed of mitochondria with degenerating inner membranes. Similar to the work of Shinzawa et al. (2008) the size and prevalence of these structures increased over the course of disease progression. Observations of the phospholipid content of these neurons led to a proposal of insufficient membrane remodeling in PLAN, possibly due to a deficit in the removal of oxidized lipid sidechains (Beck et al., 2011).

Similar deficits in mitochondrial function were seen in a drosophila knockout model of the PLA2G6 homologue of iPLA2β (Kinghorn et al., 2015). These flies showed impaired locomotor activity as measured by a climbing test and reduced survival rates (Kinghorn et al., 2015). This was accompanied by widespread neurodegeneration, abnormal mitochondria with fragmented and swollen cristae as well as retinal abnormalities (Kinghorn et al., 2015). This mitochondrial dysfunction was shown to precede morphological abnormalities (Kinghorn et al., 2015). These abnormalities were also seen in follow-up experimentation performed on fibroblasts collected from two patients with a genetically confirmed diagnosis of PLAN (Kinghorn et al., 2015). These fibroblasts showed, decreased mitochondrial membrane potential, increased generation of ROS accompanied by increased mitochondrial lipid peroxidation (Kinghorn et al., 2015). This model provides further evidence of deficiency in iPLA2β being associated with mitochondrial dysfunction and structural abnormalities.

Though iron accumulation was also notably absent in these models, a series of shared morphological characteristics were observed in the work of Beck et al. (2011), Shinzawa et al. (2008), and Kinghorn et al. (2015). Collectively these studies provide evidence that a deficiency in iPLA2β leads to the formation of abnormal spheroid
structures, which appear to form in association with swollen, dysfunctional mitochondria. The formation of axonal spheroids is also a frequently seen hallmark of PLAN in patients (Gregory et al., 2009; Gregory & Hayflick, 2013; Hogarth, 2015). Despite these promising findings, investigations of PLAN in these models is limited by their failure to capture key aspects of the disease phenotype. These models fail to robustly show early-onset neurodegenerative symptoms; either in the presence or absence of iron accumulation.

1.2. Pantothenate Kinase Associated Neurodegeneration (PKAN)

1.2.1. Genetic and Molecular Basis of PKAN

PKAN makes up the largest proportion of cases originally classified as classical HSS (Hayflick et al., 2003; Hogarth, 2015) It is caused by mutations in \textit{PANK2}, the gene encoding an isoform of pantothenate kinase, which is responsible for catalyzing the conversion of pantothenate into 4-phosphopantothenate; the first step in the production of coenzyme A (CoA) (Zhou et al., 2001). See Figure 1-2 for an overview of this reaction. The specific isoform mutated in PKAN, pantothenate kinase-2 (PANK2), shows a ubiquitous expression pattern throughout the body, but is more dominantly expressed in both the retina and BG than other isoforms of pantothenate kinase (Zhou et al., 2001). Deficiency in PANK2 has been predicted to result in a reduction in downstream secondary metabolites including CoA (Zhou et al., 2001). CoA is involved in numerous metabolic pathways including the citric acid cycle and fatty acid metabolism as well as cholesterol and sphingolipid synthesis (Rouault, 2013). Due to the ubiquitous nature of CoA throughout the cell, defects in PANK2 have the potential to have widespread effects in the cell ranging from mitochondrial dysfunction to impaired membrane synthesis.

\textbf{Figure 1-2}. Enzymatic Activity of pantothenate kinases, such as pantothenate kinase 2; which is implicated in Pantothenate Kinase-Associated Neurodegeneration (PKAN).
1.2.2. Symptoms and Presentation of PKAN

PKAN can be divided into an early-onset and rapidly progressing classic form of PKAN as well as a later-onset and more slowly progressive atypical form of PKAN (Hayflick et al., 2003), though in reality many cases exist on a spectrum between these two groupings. An absolute genotype-phenotype relationship has been difficult to determine for PKAN, but it appears that null mutations are associated with classic PKAN; early-onset and rapid progression of disease, whereas mutations predicted to have some residual function result in later onset and slower progression of the disease (Gregory et al., 2009). This relationship between the inactivating mutations and the onset and severity of disease has previously been proposed in PLAN (Engel et al., 2010). The classical presentation of PKAN represents the majority of cases and is typically in early childhood with an onset prior to 6 years of age and rapid disease progression (Gregory et al., 2009; Hayflick et al., 2003). Gait and postural difficulties due to dystonia as well as developmental delay are common early signs of PKAN (Gregory et al., 2009; Hayflick et al., 2003; Hogarth, 2015; Levi & Finazzi, 2014). Additional presentation includes dysarthria, spasticity and retinal degeneration (Gregory et al., 2009; Hayflick et al., 2003; Hogarth, 2015; Levi & Finazzi, 2014).

For atypical PKAN the age of onset is later; around 13 to 14 years of age, and the disease progression is slower (Gregory et al., 2009). A larger degree of heterogeneity is seen in the presentation of atypical PKAN, but early signs include speech difficulties, psychiatric disturbances and cognitive decline (Gregory et al., 2009; Levi & Finazzi, 2014). Atypical PKAN also features less severe and slower progressing motor symptoms than are seen in classic PKAN (Gregory et al., 2009; Levi & Finazzi, 2014).

1.2.3. Pathophysiology of PKAN

The pathology of PKAN is predominantly within the central nervous system (CNS) and is focused in the BG. Both iron accumulation and degeneration targeted primarily in the GP are hallmarks of PKAN; targeting of the SN is also frequently seen. Further understanding of the histopathological presentation of PKAN was provided by the work of Kruer et al. (2011) in a group of genetically confirmed cases. Highlights of these findings
include the visualization of two types of spheroid structures inside the BG; including a population of abundant degenerating neurons as well as a smaller population of neuroaxonal spheroids present in the axons of dystrophic neurons. Investigation of the iron deposition inside the GP by PERLs stain revealed perivascular and astrocytic iron deposits as well as cytoplasmic increases of iron inside neurons (Krueer et al., 2011). Interestingly, a reduction in iron content was seen inside degenerating neurons, though ferritin staining revealed that a notable portion of this iron is not associated with ferritin indicating that degenerating neurons may still be seeing a rise in freely reactive intracellular iron (The Labile Iron Pool or LIP). The exact link between a deficiency of PANK2, neurodegeneration of the BG and iron accumulation has yet to be determined.

1.2.4. Models and Research Efforts in PKAN

Preliminary understanding of the function of PANK2 was demonstrated in the work of Leonardi et al. (2007). They showed that PANK2 is regulated by CoA levels. They also showed its function as a mitochondrial sensor of CoA demand for β-oxidation (Leonardi et al., 2007).

Investigations by Kuo, Hayflick, and Gitschier (2007) in a mouse model deficient in PANK2 revealed retinal degeneration and defects in spermatogenesis. A follow-up study in mice which had been deprived of pantothenic acid revealed similar symptomology (Kuo et al., 2007). This is indicative that these symptoms are likely due to a deficiency of secondary metabolites downstream of pantothenic acid. Further investigation of these mice revealed mitochondrial targeting of PANK2 and accompanying defects in mitochondrial function including a global failure of the mitochondrial bioenergetic performance, a reduction in ATP production and altered mitochondrial membrane potential (Brunetti et al., 2012). These abnormalities in mitochondrial function were accompanied by histological signs in PANK2−/− mice including swollen mitochondrial with aberrant cristae and altered mitochondrial matrix structure (Brunetti et al., 2012). Notably no neurological symptoms or iron accumulation was seen in these studies.
However, these mice did develop weight loss and neurological symptoms including a severe motor deficit when exposed to a ketogenic diet (Brunetti et al., 2014).

A *drosophila* model with a mutated form of the *drosophila PANK2* homologue, *fbl*, presented with a deficiency in CoA, neurodegenerative symptoms and early lethality (Wu, Li, Lv, & Zhou, 2009). These symptoms were rescued by the administration of pantethine (Wu et al., 2009), which reinforces the likelihood of symptoms in PKAN being generated by a deficiency of downstream secondary metabolites. Despite many investigations in models of PKAN not seeing abnormal iron accumulation, a cellular model deficient in PANK2 has shown some preliminary connection between PANK2 and iron metabolism. siRNA silencing of PANK2 in Hela cells, hepatoma HepG2 cells and neuroblastoma SH-SY5Y cells led to cytosolic iron deficiency and ferroportin (FP) upregulation (Poli et al., 2010).

Further investigation of PKAN using patient derived fibroblasts by Santambrogio et al. (2015) revealed an increase in oxidative stress based on a panel of markers. This increase in oxidative stress was accompanied by a reduction in glutathione levels. Mirroring the findings of Krue et al. (2011), it was also shown that there was an increase in the LIP inside of the mitochondria in both untreated and iron loaded conditions (Santambrogio et al., 2015). Mitochondrial function was affected in these conditions, including reduced enzymatic activity of aconitase and reduced heme production as well as impaired mitochondrial bioenergetics including reduced mitochondrial membrane potential, fragmented mitochondrial shape and reduced ATP production. This parallels the findings in models of PLAN of both mitochondrial dysfunction and accompanying structural abnormalities. Evidence for the contribution of LIP to these defects was seen by the fact that these symptoms were ameliorated by deferoxamine (DFO) chelation treatment (Santambrogio et al., 2015). As a follow-up, Santambrogio et al. (2015) reprogrammed PKAN patient derived fibroblasts into neurons using direct neuronal reprogramming. These neurons showed similar elevations in oxidative stress and reduction in mitochondrial membrane potential as was seen in the fibroblasts. Despite the lack of overt iron accumulation seen in studies of mouse and fly models of PKAN, these research efforts in primary cells, isolated from patients with PKAN, demonstrate...
that disruptions in PANK2 lead to iron dysregulation within the cell; with the potential to increase the cellular LIP. The occurrence of this rise in LIP corresponded with impaired mitochondrial function and this dysfunction was mitigated by chelation therapy, which limits the LIP. This provides evidence for the possibility that iron-related cellular damage due to increases in the LIP is occurring in PKAN even in the absence of overt iron accumulation, such as what might be visible in a PERLS stain investigation.

Recent histopathological investigations as well as work in the existing models of PKAN provide insights into the possible underlying mechanisms which may be responsible for dysfunction in PKAN. However, further research efforts are limited due to the failure of these models to completely capture the observed symptomology of PKAN. In both PLAN and PKAN, to date no model is available which robustly shows early-onset neurodegenerative symptoms; either in the presence or absence of iron accumulation. The lack of this characteristic in existing models of NBIA makes efforts to establish and characterize a more representative model of NBIA for PKAN or PLAN an important area of research.

1.3. Systemic Assessment of Neurodegenerative Disorders

Another area of ongoing research in NBIA focuses on disruptions in iron metabolism, iron accumulation as well as increases in oxidative stress and inflammation, which are all commonly observed hallmarks of neurodegeneration in both NBIA and PD. Currently, limited information is available about how these hallmarks contribute to disease progression in NBIA. This deficit is particularly pronounced regarding understanding of the systemic state of these disorders. The prevalence of systemic disruptions, such as changes in systemic iron levels and systemic iron metabolism, and their contribution to disease progression in NBIA is still largely unknown.

Though preliminary investigations using animal models (Kuo et al., 2005), primary cell cultures (Campanella et al., 2012; Santambrogio et al., 2015) as well as metabolic profiling (Leoni et al., 2012), have been performed in PKAN, the systemic state in PKAN as well as other NBIA disorders is still poorly understood. Questions remain surrounding how
degeneration inside the CNS affects the systemic systems. Additionally, it is still unclear what contributions systemic elements, such as iron metabolism make to the iron accumulation and degeneration seen in the BG.

Improving understanding of the systemic dysfunction which occurs in these disorders is crucial for 3 major reasons. Firstly, it provides better understanding of systemic processes that may be occurring and contributing to disease progression. Secondly, it provides the opportunity to assess the systemic disease burden in patients. Finally, these investigations provide the opportunity to explore and evaluate candidate biomarkers which serve as indicators of biological processes, pathogenic process or pharmacological response to therapy (Biomarkers Definitions Working Group., 2001). In NBIA, biomarkers provide utility by characterizing the disease state, tracking its progression and gauging the impact and effect of therapy. Access to reliable blood-based systemic biomarkers would provide the opportunity to address current limitations in the tools that are currently available for the assessment of NBIA patients and for the evaluation of novel therapies in NBIA. Thus, biomarkers in NBIA are potential tools to improve the assessment of the disease state of patients, monitor disease progression and measure the impact of interventions.

1.3.1. Existing Clinical Tools in NBIA

Genetic Analysis

Due to the underlying genetic causes of all known NBIA disorders, genetic analysis represents a powerful tool in NBIA and remains the gold standard for the diagnosis of NBIA patients. A panel of genes has now been developed that can be analyzed to diagnose patients with suspected NBIA. The continued prevalence of idiopathic NBIA cases makes it likely that the number of genes which are identified as causative in NBIA will continue to expand improving the utility of this approach. Recent advances in genetic targeting, sequencing and bioinformatic analysis tools have enabled relatively rapid and easy diagnosis using this gene panel.
Brain Imaging (Magnetic Resonance Imaging (MRI) and Positron Emission Topography (PET) Scans)

MRI and PET scans are widely used in the diagnosis and assessment of numerous neurological disorders such as PD, Alzheimer’s disease, Huntington’s disease and NBIA. They offer the unique ability to assess the exact functional state of the brain. In NBIA MRI is used to determine the region of the brain affected based on gross structural changes or degeneration. Additionally, T2* or R2* imaging is used to assess the extent of iron accumulation as the disease progresses. PET scans provide utility by measuring the functionality of specific population of neurons, such as the dopaminergic neurons of the substantia nigra pars compacta (SNc). See Agarwal et al. (2012), for an example of MRI and PET scan assessment of a patient with PLAN.

Clinical Rating Scales

The final tool that is frequently used in NBIA is clinical rating scales such as the Unified Parkinson’s Disease Rating Scale (UPDRS) (Goetz et al., 2008) and Barry-Albright Dystonia (BAD) rating scale (Barry, VanSwearingen, & Albright, 1999). Additional scales such as Pediatric Quality of Life Inventory (PedsQL™) (Varni, Seid, & Kurtin, 2001) are used to assess quality of life in patients. One final scale, the Global Deterioration Score (GDS), is utilized to measure cognitive decline associated with neurodegenerative disease progression (Reisberg et al., 1982).

Collectively these tools provide strong clinical utility in the diagnosis of patients. Furthermore, they can be used in assessment of motor symptoms in patients as well as tracking the ongoing neurodegenerative progression inside of the brain. Limitations in these tools due to difficulty in administration, expense or training required and the poor capture of the systemic state of patients still need to be addressed. Due to their relatively low cost and ease of access, systemic blood-based biomarkers are well suited to complement these existing methods and provide additional utility in the assessment of NBIA.
1.3.2. Evidence for Systemic Changes in NBIA

A growing body of evidence exists that supports the pursuit of blood-based biomarkers in NBIA. As demonstrated by numerous research studies and investigation of patients with NBIA defects in the proteins iPLA2β and PANK2 lead to severe and debilitating changes within the CNS. As is the case in other neurodegenerative disorders such as PD, these defects selectively target areas of the BG for degeneration; particularly the GP and SN. Recent studies are also beginning to highlight that the degeneration of the BG in NBIA is accompanied by changes outside of the CNS.

Cellular studies in both fibroblasts (Campanella et al., 2012; Santambrogio et al., 2015) and erythrocytes (Siegl et al., 2013) demonstrate the potential for wide ranging systemic defects in NBIA. Investigation of primary fibroblasts isolated from patients with PKAN revealed increased oxidative stress as well as cellular defects in iron handling (Campanella et al., 2012). Further defects were seen in a follow-up study which demonstrated an elevation in the mitochondrial LIP as well as impaired mitochondrial bioenergetics and heme production accompanied by alterations to mitochondrial morphology (Santambrogio et al., 2015). Defects were also seen in some erythrocytes found in patients with PKAN (Siegl et al., 2013). Specifically, the morphology and activity of circulating erythrocytes is modified; including abnormal lipid content and calcium uptake (Siegl et al., 2013). In addition to these cellular investigations, systemic metabolic profiling in patients with PKAN showed global defects in cholesterol, lipid and bile metabolism; suggesting that defects in CoA production due to PANK2 mutations may impact the downstream synthesis of lipids reliant on CoA precursors (Leoni et al., 2012). Finally, investigation of a mouse model of PKAN revealed that defects in spermatogenesis also occur in PKAN (Y.-M. Kuo et al., 2005). Collectively, these studies highlight the potential for systemic disruptions in NBIA. A wide range of areas are shown to be impacted including fibroblasts and circulating erythrocytes as well as global defects in fatty acid synthesis.
1.3.3. Further Systemic Disruptions

The metabolic profiling performed by Leoni et al. (2012) provided preliminary evidence of large-scale systemic disruptions occurring in PKAN, that may also be present in other NBIA disorders. In spite of these efforts a number of hallmarks of neurodegeneration within the CNS in NBIA patients remain poorly explored or unexplored systemically. These included disruptions in iron metabolism as well as increases in oxidative stress and inflammation. The prevalence of systemic disruptions in these areas and their contribution to disease progression in NBIA is still largely unknown.

Further evidence which supports the hypothesis that systemic abnormalities occur in these areas as part of the disease progression in NBIA can be found in investigations of PD. Due to its greater prevalence, numerous investigations into systemic changes which accompany neurodegeneration and iron accumulation of the BG have been performed in PD (Alberio et al., 2013; Andican et al., 2012; Chen et al., 2009; Chen, O’Reilly, Schwarzschild, & Ascherio, 2007; Saracchi, Fermi, & Brighina, 2014; Wong et al., 2010). These studies highlight that oxidative stress and an inflammation, which are notable hallmarks of degeneration in the CNS of patients with PD (Adibhatla & Hatcher, 2010; Mariani, Polidori, Cherubini, & Mecocci, 2005; Nagatsu & Sawada, 2005; Niranjan, 2014; Núñez et al., 2012) are also elevated systemically. The state of systemic iron levels and systemic iron metabolism has been less thoroughly investigated in PD, but studies by Logroscino et al. (1997), Tórsdóttir, Kristinsson, Sveinbjörnsdóttir, Snaedal, & Jóhannesson, (1999), Madenci, Bilen, Arli, Saka, & Ak, (2012) and Hegde et al. (2004) show the potential for systemic dysfunction. Systemic biomarker review studies have also highlighted additional candidate biomarkers in PD including fibrinogen, transthyretin, DJ-1, alpha synuclein and serum amyloid P (Alberio et al., 2013; Chahine, Stern, & Chen-Plotkin, 2014; Saracchi et al., 2014). This body of evidence compiled from studies of NBIA and PD provides a strong rationale for the possibility of a systemic oxidative and inflammatory burden in NBIA. As well as the possibility of altered systemic iron trafficking as a contributing factor in NBIA. Further research is warranted to confirm this possibility.
1.4. Overview

Addressing limitations in currently available animal models of NBIA as well as improving understanding of the systemic state of NBIA disorders are the primary focuses of this thesis. Chapter 1 outlines the two NBIA disorders which are the focus of this investigation: PLAN and PKAN. This includes elements of their underlying genetic and molecular mechanisms, the pathophysiology and presentation of these disorders and highlights of current research efforts in NBIA. Additional information in relation to systemic dysfunctions in NBIA and available treatment avenues for these disorders are highlighted. Chapter 2 provides an overview of the key systems that are implicated in NBIA both systemically and within the CNS. This includes elements of both systemic iron metabolism and iron metabolism within the CNS as well as oxidative stress, inflammation and mitochondrial dysfunction. The final topic covered is the function and circuitry of the BG. Understanding of these elements is fundamental to appreciating the work presented in the subsequent two chapters.

Chapter 3 focuses on the further characterization of an existing mouse model of PLAN originally outlined by Strokin et al. (2012). The results of an investigation into the degeneration of the SN and the accompanying motor symptoms in these mice are presented. Due to the prominent early-onset neurodegeneration seen in these mice, this investigation was conducted based on the hypothesis that significant degeneration of the BG occurs in B6.C3-Pla2g6^m1J/CxRwb mice including a loss of dopaminergic function within the SN. Additionally, it was hypothesized that similar to PLAN patients these mice would also show abnormal iron metabolism resulting in iron accumulation within the BG. To address these hypotheses this investigation assessed the previously reported motor symptoms and weight loss in these mice, measured the dopaminergic function based on tyrosine hydroxylase expression in these mice as well as the state of GABAergic neurons within the substantia nigra pars reticulata (SNr) and evaluated the SN and other midbrain areas for the presence of overt iron accumulation.

In Chapter 4 and Chapter 5 an investigation was performed into systemic markers of iron metabolism, oxidative stress and inflammation as well as additional markers
highlighted by recent investigations in PD. This investigation was based on the hypotheses that I) Disruptions in the iron metabolism of the CNS that leads to visible iron accumulation in NBIA extends to changes systemic iron metabolism and trafficking. II) Significant increases in oxidative stress and inflammation occur systemically as part of the disease processes in PKAN, III) Abnormalities occur in lipid metabolic proteins due to mutations in lipid associated enzymes in NBIA disorders and IV) Due to the similarity between NBIA and PD, biomarkers related to neurodegeneration previously proposed in PD are also strong candidates as clinical blood-based biomarkers in NBIA. Based on these hypotheses this investigation evaluated a large panel of markers that evaluate disease contributing processes such as abnormal iron metabolism, assess the systemic disease state including oxidative stress, inflammation as well as lipid metabolism and potentially relate to disease onset and severity. The preliminary stage of this investigation focused on a single patient with PLAN. This was followed by an expanded secondary stage which explored similar markers in a group of 30 patients with PKAN. The results of these investigations are presented in Chapter 4 and Chapter 5 respectively.

For the final portion of this project, the clinical utility of the systemic markers which were part of systemic investigations into NBIA was assessed by measuring them over the course of trials of two novel therapies in NBIA: antioxidant therapy and chelation therapy. Theses investigations were similarly structured to the initial systemic investigation into the systemic state of NBIA. The same panel of markers was used to evaluate changes in disrupted systems in response to therapy, assess the impact of therapy on the systemic disease burden and validate the clinical utility of markers based on their change over the course of therapy in relation to measures of disease severity. In Chapter 4, the first stage of this investigation which focused on the evaluation of chelation therapy with the iron chelator deferiprone (DFP) and antioxidant therapy with alpha-lipoic acid (ALA) and \textit{n}-acetyl cysteine (NAC) in a single patient with PLAN is presented. Finally, the second stage of this investigation, which was an expansion to include the panel of 30 PKAN patients as they were treated with DFP, is outlined in Chapter 5. In both
of these studies the markers were explored as potential markers of the pharmacological response to therapy with the iron chelator DFP.
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Chapter 2. The Systems Implicated in the Pathogenesis and Presentation of Neurodegeneration with Brain Iron Accumulation

2.1. Implicated Systems in NBIA

Dysfunction in a number of different systems is implicated in the progression and presentation of NBIA. Elements of both systemic iron metabolism as well as iron metabolism inside of the CNS are involved in the abnormal iron accumulation which is seen in patients with NBIA disorders. Furthermore, iron is a likely contributor to the ongoing damage causing degeneration of the BG. Knowledge of its regulation and handling intracellularly and extracellularly is crucial to appreciating the dysfunction which occurs in NBIA. The damage caused by poorly regulated iron is predominantly in the form of reactive iron chemistry, which generates excess reactive oxygen species. This damage has been proposed to selectively target and impair mitochondrial function both NBIA and PD (Rouault 2013). Signs of this damage include chronic elevations in oxidative stress and inflammation. A final aspect which is central to NBIA is the function and circuitry of the BG; which is the primary target of degeneration in these disorders. This review provides an overview of these systems which are central to the disease state in NBIA. Understanding of these systems is fundamental to appreciating the work presented in the subsequent two chapters.

2.2. Iron Metabolism and Oxidative Stress

2.2.1. Iron Utilization

The presence of iron is a requirement for nearly all known forms of life; it is of fundamental importance within biological systems due to its ability to perform redox cycling between the ferric (Fe³⁺) and ferrous (Fe²⁺) state which gives iron the powerful ability to catalyze reactions (Crichton 2016). This ability is harnessed biologically by numerous iron-containing enzymes. These iron-containing enzymes can be separated into 3 major groups. In the first group iron is bound inside of a porphyrin ring, which is known as heme. An example of these haemoproteins is the oxygen carrier hemoglobin. Another common functional arrangement of iron is complexed with sulphur atoms inside of an
iron-sulphur cluster (Fe-S) cluster (ISC), such as in the iron response element binding protein (IRP) which regulates intracellular iron levels (Rouault 2006; Wallander, Leibold, and Eisenstein 2006). Notably the production of heme and ISCs are mitochondrially dependent and consume the majority of cellular iron (Crichton 2016). The third group, Non-Heme-Non-Fe-S proteins are much less recognized and more poorly understood. An example of one of these proteins is the enzyme tyrosine hydroxylase (TH), which is a homotetramer that contains an iron atom in each of its four subunits (Nagatsu 1995). TH is responsible for the conversion of L-Tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) (Nagatsu 1995); the rate limiting step in the production of dopamine. Additionally, a final group of iron containing proteins is responsible for ensuring the supply and distribution of iron throughout the body. These include iron transport channels and receptors such as transferrin receptor 1 (TfR1) and the divalent metal ion transport 1 (DMT1) as well as ferroportin (FP), which governs cellular iron export. The predominant carrier protein of iron in the body is the transport protein transferrin (Tf). Haptoglobin and hemopexin bind and transport free hemoglobin and heme respectively. Finally, iron storage is largely carried out by iron sequestering protein ferritin, which is made up of 2 subunits, H-ferritin and L-ferritin.

2.2.2. Reactive Oxygen Species and Fenton Chemistry

As evidenced by the wide array of proteins noted above, the powerful catalytic ability of iron is essential for numerous biological functions. However, iron also catalyzes reactions which are highly undesirable in biological systems due to the reactive products generated which in turn cause damage to cellular DNA, proteins and lipids. These reactive products can be broadly categorized as reactive oxygen species (ROS) which can be further subdivided into individual physiologically relevant ROS including superoxide (O$_2^-$), hydroxyl radicals (OH•) and hydrogen peroxide(H$_2$O$_2$) (Koskenkorva-Frank et al. 2013).

2.2.3. Antioxidant Balance

During normal physiological function these ROS are generated from cellular metabolism and also play a role in cellular signaling and the regulation of a diverse range
of functions (Dröge 2002). In order to maintain these cellular functions while minimizing the risk of unintended cross-reactivity of ROS, both enzymatic and non-enzymatic controls are used to maintain a narrow concentration range of these species. Enzymatic controls include superoxide dismutase, which converts superoxide into hydrogen peroxide, catalase which converts hydrogen peroxide to water and oxygen, and glutathione peroxidase, which utilizes glutathione to convert hydrogen peroxide to water. Common non-enzymatic antioxidants include ascorbate (Vitamin C), glutathione, \( \alpha \)-tocopherol (Vitamin E), NAC and ALA (Dröge 2002; Koskenkorva-Frank et al. 2013).

2.2.4. Reactive Iron Chemistry

As noted above, the presence of excess iron catalyzes the production of excess ROS. This is particularly due to its ability to generate hydroxyl radicals through the Fenton Reaction. In this reaction ferrous iron reacts with hydrogen peroxide to form hydroxyl radicals (Prousek 2007). Hydroxyl radicals are extremely reactive, freely reacting with cellular DNA, proteins, carbohydrates and lipids damaging their structure through the addition of hydroxide groups (-OH) (Koskenkorva-Frank et al. 2013). This can be further exacerbated by the actions of the accompanying Haber-Weiss reaction, which utilizes superoxide to convert ferric iron to ferrous iron; allowing it to further react via the Fenton reaction (Koskenkorva-Frank et al. 2013). There is no native enzymatic reaction for the conversion of hydroxyl radicals, as is the case for other ROS, thus cells have a limited ability to mitigate the damage caused by hydroxyl radicals, but use low-molecular weight non-enzymatic antioxidants such as alpha-tocopherol, which react with hydroxyl radicals, lowering damage to cellular macromolecules.

2.2.5. Lipid Peroxidation

Under conditions which lead to the generation of excess ROS, such as in the presence of excess iron, the enzymatic antioxidant defenses present in the cellular environment are overwhelmed allowing ROS to oxidatively attack other cellular components. Non-enzymatic antioxidants serve as a second line of defense against this damage, acting as preferential sites of oxidation for these species and limiting the damage
that is caused to the cell. Dependent on the number and nature of available non-enzymatic species excess ROS has the potential to overwhelm available antioxidant substrates which leads to both localized oxidative damage at the site of their generation, such as inside of the mitochondria, as well as diffuse oxidative damage as the ROS travel intracellularly and extracellularly. One of the primary targets of this damage is cellular membranes and other lipid species (Ayala, Muñoz, and Argüelles 2014; Yin, Xu, and Porter 2011). Phospholipids, cholesterol and glycolipids, particularly those which are polyunsaturated are all common targets of oxidative attack (Ayala, Muñoz, and Argüelles 2014; Yin, Xu, and Porter 2011).

One of the most commonly used biological marker of lipid peroxidation is the short aldehyde malondialdehyde (MDA) (Ayala, Muñoz, and Argüelles 2014; Karatas, Karatepe, and Baysar 2002; Marnett 1999; Del Rio, Stewart, and Pellegrini 2005; Zelzer et al. 2013). MDA is generated as the end product of the peroxidation of polyunsaturated fatty acids, such as arachidonic acid (Ayala, Muñoz, and Argüelles 2014; Del Rio, Stewart, and Pellegrini 2005). It is well established as an almost ubiquitous hallmark of oxidative cellular and tissue damage (Ayala, Muñoz, and Argüelles 2014; Marnett 1999; Del Rio, Stewart, and Pellegrini 2005; Zelzer et al. 2013). MDA also serves as a potential contributor to these disorders due to its ability to freely react with and damage the nucleotide bases of DNA as well as various proteins (Marnett 1999; Del Rio, Stewart, and Pellegrini 2005). MDA exists as two separate pools. On short timescales after it is generated, MDA exists freely, and is not bound to other cellular components, such as DNA and proteins. Free MDA is either converted to a carboxylic acid via aldehyde dehydrogenase or reacts with cellular targets. Once bound to DNA or proteins the MDA-adduct is stable over much longer timescales. Thus, these two pools of MDA: free and total MDA, are representative of acute oxidative injury and chronic oxidative injury respectively.
2.2.6. Iron Metabolism and Its Regulation

The ability of iron to catalyze redox reactions has led to it being crucial to biological functions in almost all known forms of life. The essential nature of iron however, must be balanced against its ability to catalyze the production of damaging ROS. Due to this, iron uptake, utilization and storage are highly regulated to ensure that adequate iron is available and to limit the damage caused by ROS. The majority of the iron present in the body is dedicated to the erythron and is stored in the hemoglobin of mature and developing erythrocytes. The purpose of the erythron is to fulfill the essential role of oxygen and carbon dioxide transport (Abbaspour, Hurrell, and Kelishadi 2014; Andrews 2008; Wang and Pantopoulos 2011). Notable iron stores are also found in the liver and macrophages responsible for the recycling of senescent erythrocytes and myoglobin inside muscle tissues (Wang and Pantopoulos 2011). Excess body iron is primarily stored in hepatocytes of the liver (Andrews 2008; Wang and Pantopoulos 2011). Other tissues, such as the BG also have notable iron stores (Hardy et al. 2005; Hill and Switzer 1984; Rouault 2013).

2.2.7. Iron Uptake

In individuals who are not being transfused, the intake of iron is entirely though the diet. This intestinal iron absorption, which occurs primarily in the proximal portion of the duodenum, is a key site of iron regulation because of the lack of a well defined mechanism for iron excretion. Typically, outside of blood-loss, iron is only lost from the body due to the natural turnover and loss of enterocytes or other mucosal cells and skin cells (J. Wang and Pantopoulos 2011). Inside the lumen of the small intestine specialized villi projections on the apical side of enterocytes are responsible for the uptake of iron. Ferric iron is converted to the ferrous state by ferrireductases and uptake occurs using the divalent metal ion transporter (DMT1) (Andrews 2008). Once inside enterocytes iron is primarily distributed to one of two fates. Expression of the iron exporter ferroportin (FP) on the basolateral membrane allows for the export of iron out of enterocytes and into the blood. Ferrous iron is freely exported out of enterocytes by FP. As part of this process it is converted to a ferric state by the membrane bound ferroxidase hephaestin,
which is situated in the membrane to directly interact with FP, or by the homologous plasma protein, ceruloplasmin (J. Wang and Pantopoulos 2011). Iron which is not exported is stored inside ferritin. Ferritin is found in almost all eukaryotic cell types, and a small pool of ferritin is excreted into the blood mostly as L-ferritin, which contains little iron, but is often used as a clinical indicator of iron storage levels in the body (Andrews 2008). Iron which is not exported from enterocytes remains stored in ferritin. This iron is lost during the regular turnover of enterocytes which is typically a few days (Ganz 2011).

Once it is converted to the ferric state, iron exported from enterocytes is largely bound to the primary blood iron transport protein transferrin (Tf). TF binds to Tfr1 and is taken into the cell by receptor-mediated endocytosis (Andrews 2008; Huebers and Finch 1987; J. Wang and Pantopoulos 2011). The endosome is acidified which facilitates the release of iron from transferrin, where it is reduced to a ferrous state before being transported out of the endosome by DMT1 (Andrews 2008; J. Wang and Pantopoulos 2011). Upon its release iron is then transported to the mitochondria or other cellular locations for its utilization.

2.2.8. Iron Regulation

Though a number of other players do exist, the workhorse of iron regulation is the peptide hormone hepcidin. Hepcidin is produced by hepatocytes in response to high plasma iron levels and tissue iron storage (Ganz 2011; Haase 2013; Kautz et al. 2014). It causes the internalization of FP and a result decrease in the iron released into the blood from tissue storage as well as a decrease in iron absorption by enterocytes (Ganz 2011). In order to meet the specific iron demands of various cell types throughout the body a dynamic intracellular iron regulatory system also exists. A pair of iron regulatory proteins (IRP1 and IRP2) bind to a specific motif in the untranslated of mRNAs for iron trafficking proteins known as an iron regulatory element (IRE). Depending on the location of the IRE within a proteins transcript, IRPs can upregulate or downregulate its production in response to low iron levels (Rouault 2006; Wallander, Leibold, and Eisenstein 2006; J.
Wang and Pantopoulos 2011). By dynamically regulating the production of proteins such as TfR1 and ferritin cellular iron uptake and storage is regulated by the IRP-IRE system.

2.2.9. Iron Pools

These robust regulatory systems ensure that during normal iron trafficking iron is contained within three pools. It is either I) Utilized inside iron containing proteins, II) Sequestered in ferritin, or III) Bound and transported by transferrin. In all three of these states the reactive ability of iron is regulated by being in the ferric state, being part of a protein complex or both. This limits the ability of iron to catalyze reactions which cause damage to the cellular environment. Under normal physiological conditions with fully functioning iron regulation, very little iron is not in one of these three states which represents a fourth and final pool. This final pool of iron is known as non-transferrin-bound iron (NTBI). The saturation of the transferrin in the plasma is typically around 30% (Brissot et al. 2012). At this saturation level the majority of iron is captured by transferrin and minimal NTBI is present, the saturation of transferrin rises and its ability to capture and transport available iron decreases. Under these conditions significant NTBI is generated. Additional proteins and other lower molecular weight plasma components that are both present in high concentrations and able to bind this NTBI (Brissot et al. 2012; van der Heul et al. 1972; Hider 2002; May, Linder, and Williams 1977). Collectively with transferrin these compounds represent the total iron binding capacity (TIBC) of the blood. Any NTBI which is not bound to these other proteins and components is freely reactive and known as labile plasma iron (LPI).

2.2.10. Mitochondrial Basics in Disease

The predominant intersection between iron trafficking, iron utilization and elevated cellular oxidative stress is inside the mitochondria, which contains systems responsible for ATP generation, the regulation of cell survival, apoptosis and the production of steroid hormones as well as the formation of heme and ISC containing proteins (Lin and Beal 2006; Huang, Lane, and Richardson 2011; Lill 2009). These mitochondrial systems also contain the majority of oxidative pathways in the cell;
including multiple redox sites capable of generating extremely reactive species such as superoxide anions (Andreyev, Kushnareva, and Starkov 2005). There is a growing body of evidence implicating mitochondrial dysfunction in numerous diseases including the neurodegenerative disorders Alzheimer’s disease, PD and Huntington’s disease. A recent review by Lin and Beal (2006) highlights the contributions that mitochondrial dysfunction can make to neurodegeneration disorders and outlines how this dysfunction may be a shared hallmark of this diverse group of disorders. This disrupted mitochondrial state has also been implicated in NBIA. The function of a number of the proteins involved in NBIA converges on the mitochondria: including PANK2 and iPLA2β (Levi and Finazzi 2014; Rouault 2013). Abnormalities in mitochondrial shape and function caused by deficits in these proteins are frequently noted in both PKAN (Kruer et al. 2011) and PLAN (Hogarth 2015). Further evidence for this hypothesis is provided by the verification of mitochondrial dysfunction and abnormalities in numerous disorders of PLAN (Beck et al. 2011; Kinghorn et al. 2015; Shinzawa et al. 2008) and PKAN (Brunetti et al. 2012; Santambrogio et al. 2015).

2.2.11. Mitochondrial Iron Metabolism

The mechanisms responsible for the regulation of mitochondrial iron levels are relatively poorly defined, but it is likely that a signaling system, such as the Friedrich’s ataxia associated protein frataxin, exists to ensure that sufficient mitochondrial iron is available for heme and ISC formation. Studies in *Saccharomyces cerevisiae* have demonstrated a strong relationship between ISC synthesis and the regulation of mitochondrial iron metabolism (Lill and Mühlenhoff 2008). When ISC synthesis is reduced or impaired there is an increase in cellular iron uptake and a change in iron distribution within the cell; favouring mitochondrial deposition at the expense of cytoplasmic iron content (Lill and Mühlenhoff 2008). This process is well established and has been seen in other neurological and movement disorders, including Friedrich’s ataxia; where the loss of the mitochondrial protein frataxin impairs ISC synthesis and leads to mitochondrial iron accumulation (Vaubel and Isaya 2013). Mitochondrial dysfunction and mitochondrial iron overload have also been observed in PD (Horowitz and Greenamyre 2010;
Mastroberardino et al. 2009). Overall this evidence highlights that, in order to preserve essential ISC production within the cell, a mechanism exists which diverts cellular iron to the mitochondria (Isaya 2014; Núñez et al. 2012). Ultimately this would result in a deficiency in cytosolic iron and subsequent accumulation of iron and increased oxidative stress within the mitochondria (Isaya 2014; Núñez et al. 2012) which is seen in models of NBIA (Santambrogio et al., 2015).

2.2.12. Iron and the Blood-Brain Barrier

The mechanics of the movement of iron into and out of the CNS are poorly understood relative to the systemic trafficking of iron. However, some basic aspects of brain iron metabolism have now been determined. Functionally, the movement of most nutrients into the brain; including iron, is limited by the blood-brain barrier (BBB) (Ballabh, Braun, and Nedergaard 2004; Bradbury 1997). The BBB is composed of brain endothelial cells (BECs) which use a combination of cytoskeletal elements such as tight junctions, claudins and occludins to limit and regulate the movement of nutrients into the interstitial fluid of the brain (Ballabh, Braun, and Nedergaard 2004). Additionally, a strong associate between astrocytes and the BBB is seen and has been proposed to be necessary for the proper maintenance and function of this barrier (Ballabh, Braun, and Nedergaard 2004). Notably the composition of the blood-brain barrier also includes the basal lamina associated with the BECs as well as pericytes, which provide further regulation of blood-flow and BBB permeability (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010). To enter the brain, iron must pass through the luminal barrier membrane of these endothelial cells and subsequently be transported out of the endothelial cells on the abluminal side into the interstitial fluid. The most likely, and most robustly proven method for iron movement across the luminal membrane is by the receptor mediated endocytosis of transferrin-bound iron via the TfR (Ballabh, Braun, and Nedergaard 2004; Bradbury 1997; Rouault 2013). FP expression has been reported in the endothelial cells of the BBB in some studies (McCarthy and Kosman 2013; Wu et al. 2004) and remains the most likely method for iron export from BBB endothelial cells as no significant alternate pathways for iron export are known to exist. This is supported by the observation of the expression of
hephaestin by BECs (McCarthy and Kosman 2013) as well as in the foot processes of BBB associated astrocytes (Rouault and Cooperman 2006) which would be necessary to convert the ferrous iron to a ferric state for transferrin transport within the CNS. The astrocytes which are found in association with the BBB are also key in the regulation of iron in the brain. They have been shown to be able to take up iron using a DMT1 dependent mechanism (Dringen, Bishop, Koepppe, Dang, & Robinson, 2007) as well as the ability to take up heme iron in a mechanism that has no been fully characterized (Dringen, Bishop, Koepppe, Dang, & Robinson, 2007). Astrocytes also have been shown to be readily able to store iron from a variety of sources in ferritin and to express a full complement of iron export machinery (Dringen, Bishop, Koepppe, Dang, & Robinson, 2007). The presence of iron import, export and storage machinery in astrocytes in context of their association with the BBB make it likely that astrocytes are key regulators of the delivery of iron to other cells throughout the brain (Dringen, Bishop, Koepppe, Dang, & Robinson, 2007).

Though it was originally proposed as a possible mechanism for the movement of iron into the brain, radiolabeling studies have demonstrated that systemic Tf does not transcytose the BBB and enter the brain’s interstitial fluid (Moos et al. 2006; Rouault and Cooperman 2006), it is likely recycled inside of BECs instead. Functionally, this means that the CNS Tf pool can have a different iron saturation then the systemic Tf pool. Total brain Tf levels are about 10% of serum levels (Rouault and Cooperman 2006). It has been reported that the saturation of Tf found in the CSF, is nearly complete (Burdo and Connor 2003), which is in sharp contrast to the typical saturation levels of systemic Tf of ~30% (Brissoot et al. 2012). Highly saturated Tf pools in the CSF means that a notable percentage of iron within the CNS may exist as NTBI. The mechanism for the accumulation of excess iron inside of the BG in NBIA still needs to be determined, but the highly saturated nature of Tf within the brain means that even minor increases in iron could contribute to a rise in NTBI and ultimately freely reactive LPI.
2.2.13. Cellular Iron Metabolism in the Brain

Though the specific iron regulatory systems used by the BG are still being explored, it has been shown that the intracellular iron handling system utilized systemically is largely present in the brain. This includes DMT1, FP, mitoferrin, ferritin and Tfr1 as well as IRP1 and 2 (Cooperman 2006; Rouault 2013). As is the case systemically, each CNS cell type is thus likely equipped with the regulatory machinery to control iron uptake, utilization, storage and export to meet its specific cellular requirements. It is likely that different neuronal cell types have different iron demands and regulations, however the full details have not yet been determined. This complexity is reflected in the variability of iron distribution between oligodendrocytes, astrocytes neurons and microglia. Notable iron stores are seen inside of glial cells; large amounts of ferric ferritin iron deposition are seen inside of oligodendrocytes, with lesser amounts found in both astrocytes and microglia (Connor, Menzies, Martin, & Mufson, 1990). For instance, high iron demand in oligodendrocytes is likely to meet the requirements of synthesizing and maintaining myelin (James R. Connor & Menzies, 1996). Thus, iron regulatory systems would need to reflect this variable demand and storage of iron.

In addition to these cellular systems brain iron metabolism and regulation likely has its own sets of signaling and iron sensing pathways which operate independently from systemic systems. Brain iron levels are largely maintained even in the presence of iron deficiency which largely depletes liver iron stores (Bradbury 1997). Conversely, in cases of extreme iron overload, such as those seen in hemochromatosis, excess iron accumulation in the brain is largely absent in both hemochromatosis patients and mouse models (Golub et al. 2005; Rouault and Cooperman 2006). Widespread expression of hepcidin inside the CNS has recently been demonstrated (Nemeth and Ganz 2009; Wang et al. 2010). Furthermore, CNS cells were shown to respond to hepcidin expression both in vivo and in vitro by down regulating FP expression as is seen systemically (Wang et al. 2010). This provides evidence for a functional separation of the regulation of systemic and CNS iron metabolism.
The brain represents the largest single source of oxygen demand in the body and even small depletions of oxygen availability can be devastating to the health and function of the CNS. Interestingly, high mRNA expression of the transcription factor HIF2a, which responds to hypoxia, has been shown at the BBB (Tian, McKnight, and Russell 1997). Additionally it has been shown that that BECs also respond strongly to HIF1a signaling such as in ischemic stroke (Sandoval and Witt 2008). These systems are likely in place to ensure that adequate oxygenation of brain regions is maintained, but it represents another possible mechanism by which the CNS could respond to hypoxia, iron depletion or increased iron demand independently or regardless of systemic signaling. Though the basic elements of iron metabolism inside of the CNS are beginning to be elucidated, questions remain as to the mechanism by which excess iron accumulates in NBIA. It appears that demand for iron within the CNS is able to signal independently of systemic systems. Thus, it is feasible that if iron demand is increased in the BG in NBIA, such as in the case of dysfunctional mitochondrial having impaired ISC production, the CNS would be able to signal for iron delivery across the BBB from systemic transferrin.

2.3. Inflammation

The natural role of the bodies immune system is to respond to possible sites of infection or damage. This response involves the recruitment of leucocytes and microglia to these sites to resolve these possible issues by destroying infectious agents or removing and repairing damaged tissues. A lack of resolution to this immune response leads to the onset of a chronic inflammatory state. Ongoing tissue injury and dysfunction, such as that which is seen in NBIA, initiates an immune response aimed at its resolution. One facet of this response is the non-specific release of ROS (Andersen 2004). This response increases oxidative stress and reduces the antioxidant capacity of affected cells (Khansari, Shakiba, and Mahmoudi 2009). In a vicious cycle oxidative stress can lead to tissue damage and cellular dysfunction, provoking an immune response. This response generates further oxidative stress; contributing to further cellular dysfunction. A number of cytokines are involved in mediating this process, but two which are commonly implicated in this chronic inflammation are IL-6 and TNFα (Aletaha et al. 2010; Beal 2006; Coussens and Werb 2002;
Though this chronic inflammation is often poorly resolved, signalling systems exist which are utilized by the immune system in an attempt to minimize the ongoing inflammation. One cytokine involved in these processes is the anti-inflammatory cytokine IL-10. Upregulation of IL-10 has been proposed as a possible immune response to attempt to resolve or limit chronic inflammation (Mizoguchi et al. 2002). The lack of resolution to this cycle causes ongoing chronic inflammation to be a contributor to a number of disease states. The presence of chronic inflammation has been implicated in the pathogenesis of cancer (Coussens and Werb 2002), diabetes (Dandona, Aljada, and Bandyopadhyay 2004), rheumatoid arthritis (Aletaha et al. 2010) and cardiovascular disease (Pearson et al. 2003). It is also a commonly seen hallmark of PD (Beal 2006; Andican et al. 2012; Chen et al. 2007; Nagatsu and Sawada 2005; Niranjan 2014).

2.4. **The Basal Ganglia**

The basal ganglia are a series of subcortical nuclei which are highly interconnected in a series of circuits which are crucial for the regulation of motor functions (Alexander and Crutcher 1990). Both iron accumulation and degeneration predominantly occur within the BG in NBIA (Gregory, Polster, & Hayflick, 2009; Gregory & Hayflick, 2013; Hogarth, 2015; Levi & Finazzi, 2014; Schneider, Hardy, & Bhatia, 2012). An appreciation for the selective vulnerability of the BG arises from an understanding of the high iron levels present, the complex circuitry involved and the metabolic demands of the chronic activity of many of the nuclei within the BG.

2.4.1. **Iron in the BG**

The regulation of iron movement provided by the BBB does not appear to inhibit the accumulation of iron inside of the brain. Neuroanatomical and MRI investigations have consistently revealed that significant iron deposit occurs within the brain; particularly in the form of ferric protein-bound iron (Rouault 2013). The BG, in particular the GP and SN are among the most iron rich areas of the brain, with concentrations approaching or equaling those found in the liver; the primary systemic site for iron storage.
(Hardy et al. 2005; Hill and Switzer 1984; Rouault 2013). In addition, a strong age-related increase in iron deposition is seen in the BG (Hardy et al. 2005; Hill and Switzer 1984). This iron accumulates strongly in areas the striato-pallido-nigral pathway (Francois, Nguyen-Legros, and Percheron 1981). The most heavily staining areas for iron are those that receive GABAergic innervation (Francois, Nguyen-Legros, and Percheron 1981; Hill 1985) such as the SN and GP. Interestingly, these areas are the most common targets of neurodegeneration in both NBIA and PD.

2.4.2. Basic Anatomy of the Basal Ganglia

The key nuclei of the BG include: The striatum (caudate and putamen) the GP, which can be subdivided in the GP internal segment (GPi) and the GP external segment (GPe), the SN pars reticulata (SNr), the SN pars compacta (SNc) and the subthalamic nuclei (STN) (Alexander, DeLong, and Strick 1986; Alexander and Crutcher 1990; DeLong and Wichmann 2007). Output signals from the BG are sent by the GPi and the SNr. These output signals innervate 3 main targets in the brain: the thalamus, the superior colliculus and pedunculopontine nucleus. Further connectivity of these targets is responsible for the diverse and far reaching functional influence of the BG (Lee 2007). Of critical importance to motor dysfunction in BG disorders is output to the thalamus. The thalamus innervates areas of the motor cortex with glutaminergic synapses in order to initiate movement. This activity is tightly regulated by the chronic inhibition of the thalamus, which is provided by the GPi and SNr. The far-reaching connections of the BG can be seen directly in the complexity and heterogeneity of symptoms in BG disorders such as NBIA. Within the BG these nuclei are linked together into distinct, but often interconnected circuits. An overview of the layout of the BG as well as the basic circuitry of both the direct and indirect motor pathways in the BG for the human brain is also provided for both the normal physiological state (Figure 2-1A) as well as a highlight of potential disruptions of this system in NBIA disorders such as PLAN and PKAN (Figure 2-1B).
**Figure 2-1.** Layout and circuitry of a coronal section of the human basal ganglia. Circuitry of the direct and indirect motor pathways is also highlighted. Nuclei of the basal ganglia include the substantia nigra pars compacta (SNc), the substantia nigra pars reticulata (SNr), the subthalamic nuclei (STN), the thalamus, the globus pallidus internal segment (GPI), the globus pallidus external segment (GPE) as well as the caudate and putamen which collectively make up the striatum. The motor cortex is also shown. DA indicates dopaminergic innervation from the SNc to the putamen. In their normal physiological state, the nuclei of the basal ganglia are highly interconnected including connections between the SNr, SNc and GPE as well as the Thalamus (A). Iron accumulation is typically seen in both the SN and GP (Brown Box). In NBIA disorders such as PLAN and PKAN iron accumulation in the GP and SN increases and the circuitry between the GP, SN and Thalamus are potential targets for dysfunction (Red X) (B). Figure is modified from the original created by Filippo et al., 2014.

**Dopaminergic Circuitry**

The dorsal striatum receives innervation from DN neurons of the SNc and is crucial in the regulation of motor function. Interestingly, dopamine receptors can have both excitatory and inhibitory effects. As reviewed by Sealfon and Olanow (2000), D1 receptors are typically excitatory and result in depolarization and D2 receptor are typically inhibitory and result in hyperpolarization. DN neurons from the SNc project to medium spiny neurons in the striatum. These neurons then project to the GPI (direct pathway), GPE (indirect pathway) or to the SNr. Thus, the DN inputs from the SNc to the striatum have the ability to stimulate the direct pathway (D1) and inhibit the indirect pathway (D2). This causes a disinhibition of glutamatergic neurons in the thalamus, which then activates
the motor cortex. It has long been suggested that improper balance between the activity of the direct and indirect pathway; due to disruption of the activity of the SNc, is a key factor responsible for the motor symptoms seen in BG disorders such as NBIA or Parkinson’s disease (Dauer & Przedborski, 2003).

DN neurons of the SNc exhibit strong continuous pacemaker activity. This activity is slow-spiking with frequencies in the range of 0.2-10 Hz (Surmeier, Mercer, and Chan 2005). SNc DA neurons generate action potentials in a clock-like pacemaking manner (Grace and Bunney 1983) which is central to sustaining DA levels in the innervated targets of the DA neurons such as the striatum. Due to its chronic activity, high metabolic requirements are associated with this activity and an accompanying high density of mitochondria, giving rise to oxidative stress. A large portion of these metabolic demands is the maintenance of fluctuating intracellular Ca\(^{2+}\) levels, which are necessary to sustain DA neuron activity (Chan, Gertler, & Surmeier, 2009). This pacemaker activity leads to a sustained release of dopamine onto downstream striatal targets. The neurotransmitter dopamine is synthesized as part of the same pathway responsible for the production of epinephrine and norepinephrine. TH catalyzes the rate limiting step of this synthesis (Nagatsu 1995). It is a homotetramer which relies on an association with ferrous iron inside of the active site of each subunit to catalyze the conversion (Daubner, Le, and Wang 2011; Nagatsu 1995). Due to the rate limiting nature of TH on dopamine production, high levels of TH are likely at least partially responsible for the high levels of iron seen in the SNc.

GABAergic Modulation: The Striato-Pallido-Nigral Circuit

DN neurons in the SNc receives significant input from GABAergic neurons (~70% of inputs), these inputs arise predominantly from the GPe and the SNr (Lee 2007). GABAergic neurons in the GPe and SNr are fast-spiking pacemakers, capable of sustained firing rates in excess of 50 Hz (Lee 2007; Surmeier, Mercer, and Chan 2005). As is the case with SNc DN neurons, constant activity in the GABAergic neurons of the GPe and SNr represents a significant metabolic cost. GABAergic inputs to the SNc from the SNr and
GPe have been shown to be strongly influence the activity of DN neurons on the SNc (Lee 2007). In addition to these direct connections to the SNc, it appears that the GPe also modulates the activity of the SNr. Collectively, these findings show the complex regulatory relationship of the BG. Dysfunction in any one of these nuclei have the potential to disrupt the entire system as is demonstrated in NBIA.
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Chapter 3. Characterization of a B6.C3-Pla2g6^{m1J/CxRwb} Mouse Model of Phospholipase A2G6-Associated Neurodegeneration (PLAN)

3.1. Abstract

Phospholipase A2G6-Associated Neurodegeneration (PLAN) is a rare early-onset monogenic neurodegenerative movement disorder which primarily targets the basal ganglia. Research efforts to improve understanding of disease mechanisms and test novel therapies in PLAN are currently limited by the lack of an available model which satisfactorily captures the symptomology that is observed in patients. We describe here a B6.C3-Pla2g6^{m1J/CxRwb} mouse model of Phospholipase A2G6-Associated Neurodegeneration (PLAN) which presents with serous early-onset neurodegeneration which is characteristic of the disease progression that is observed in patients. Over the course of 90 days homozygous mice present with a progressively worsening motor deficit including an overt resting tremor and impairment on a wire hang test. Fluorescent imaging revealed that this motor deficit is accompanied by a reduction in dopaminergic activity in the SNc as measured by tyrosine hydroxylase expression, which significantly correlates with the loss of motor ability in the mice. Additional fluorescent imaging with THY1-YFP revealed the formation of widespread spheroids throughout the midbrain as well as the concentration formation of spheroids with the SNr. This formation strongly mirrors the neuroaxonal dystrophy and spheroid formation observed in patients with PLAN and was demonstrated to precede the emergence of motor symptoms in the mice. PERLS/DAB staining revealed the presence of slight iron accumulation with the SN of the mice; based on a 15% increase in mean intensity, which is characteristic of PLAN. Collectively, these findings highlight B6.C3 Pla2g6^{m1J/CxRwb} mice as a useful model of PLAN. These mice present with the a severe and early-onset motor deficit, which has not been fully captured by other models of the disease. Furthermore, key hallmarks of PLAN including degeneration and iron accumulation within the BG were demonstrated in these mice. We would highly recommend this model for future research in PLAN, as to date it is the only available model which captures many of the major hallmarks of PLAN including
severe-early onset neurodegeneration, a motor deficit as well as degeneration and iron accumulation within the BG.

3.2. Introduction

Neurodegeneration with brain iron accumulation (NBIA) is a group of rare monogenic disorders. These disorders share the common hallmarks of early-onset neurodegeneration, extrapyramidal motor symptoms and the accumulation of iron inside of the BG. To date, 10 different NBIA disorders have been genetically characterized. See Appendix A for an overview of these NBIA disorders. Despite this characterization many aspects of pathophysiology of these disorders still remains to be determined. A major limitation of current research efforts into NBIA is the lack of an animal model which captures the disease presentation seen in patients. A notable feature of NBIA which is poorly represented in many of the existing disease models is the presence of prominent early-onset neurodegeneration. In many of the previous investigations in mouse models of NBIA signs of overt neurodegeneration were not seen (Beck et al., 2011; Brunetti et al., 2012; Kuo et al., 2005). A selection of models did show later onset neurological symptoms (Shinzawa et al., 2008; Zhao et al., 2011) or could be induced to undergo neurodegeneration (Brunetti et al., 2014). Collectively, these models fail to robustly show early-onset neurodegenerative symptoms; either in the presence or absence of iron accumulation. Efforts to establish and characterize a more representative model of NBIA is an active area of research.

3.2.1. Phospholipase A2G6 associated neurodegeneration (PLAN)

Phospholipase A2G6 associated neurodegeneration (PLAN) is caused by mutations in the PLA2G6 gene encoding the calcium independent phospholipase A2G6 (iPLA2β). It has proposed roles in membrane dynamics and homeostasis through phospholipid remodeling, as well as leukotriene and prostaglandin synthesis, apoptosis and inflammation (Morgan et al., 2006; Schneider, Bhatia, & Hardy, 2009; Sun et al., 2010). Pathogenic mutations found in PLAN generate enzymatically inactive isoforms which inhibit the actions of the phospholipase and render it inactive (Morgan et al., 2006). These
defects in iPLA2β have the potential to have wide ranging effects in the cellular environment.

The presentation and symptomology of PLAN can be broadly separated into 3 distinct disorders, infantile neuroaxonal dystrophy (INAD), a typical neuroaxonal dystrophy (aNAD) and a later onset progressive dystonia-parkinsonism syndrome which has also been classified as a form of PD (PARK14). INAD presents early in life, between 6 months and 3 years of age, and has a rapid and devastating progression (Gregory & Hayflick, 2013; Hogarth, 2015). aNAD represents a small subset of INAD cases with a later emergence and delayed progression. PARK14 is a progressive dystonia-parkinsonism syndrome which manifests in late adolescence or early adulthood (Hogarth, 2015). The shared classification of this presentation of PLAN as both NBIA and PD provides evidence for the strong parallels between NBIA and PD. The symptomatic presentation of these three subtypes of PLAN outlined in full in Chapter 1.

Iron accumulation inside the basal ganglia is often, but not universally seen in INAD and aNAD. This accumulation is predominantly in the GP, but it is also frequently seen in the SN on MRI (Gregory & Hayflick, 2013; Hogarth, 2015). Iron accumulation is seen with variable severity in the GP and SN of nearly all of the patients with PLAN/PARK14 (Gregory & Hayflick, 2013; Hogarth, 2015). Optic and cerebellar atrophy are also seen in INAD and aNAD (Gregory & Hayflick, 2013; Hogarth, 2015). Dystrophic neuroaxonal spheroids characteristic of PLAN are found in the brainstem, peripheral nerves, BG and spinal cord of patients (Hogarth, 2015).

It is likely that these three sub-classifications of PLAN; which show a range of age of onset as well as severity of progression, are related to the nature of the inactivating mutations in iPLA2β found in each patient. This relationship between the inactivating mutations and the onset and severity of disease has previously been proposed in Pantothenate Kinase-Associated Neurodegeneration (PKAN), another form of NBIA (Gregory, Polster, & Hayflick, 2009). A recent study revealed that the nature and location of mutations in the PLA2G6 gene may be responsible for the large degree of
heterogeneity between the various forms of PLAN (Engel, Jing, O’Brien, Sun, & Kotzbauer, 2010). The severity of impairment of iPLA2β function may be directly related to the presentation of PLAN, with a more severe mutation resulting in the more rapid onset and quicker progression of INAD, which also sees a more widespread pathology and a later onset dystonia-parkinsonism phenotype seen in mutations with less impact on iPLA2β catalytic activity. This relationship could explain much of the heterogeneity seen in the presentation of PLA2G6 mutations in various mouse models of PLAN. In light of this, a full characterization of proposed mouse models of PLAN should be undertaken in order to determine their accuracy in capturing the pathophysiology and presentation of the various forms of PLAN.

3.2.2. Further Investigations in B6.C3-Pla2g6<sup>m1J/CxRwb</sup> Mice

Recently a novel mouse model of Phospholipase A2G6-Associated Neurodegeneration (PLAN) has been highlighted by the work of Strokin, et al. (2012), which notably features prominent early-onset neurodegeneration as young as 50 days of age which is visible by an accompanying motor deficit. The presence of neurodegenerative symptoms which more closely match those seen in NBIA patients makes this model a promising candidate for research into NBIA. The work done by Strokin et al. (2012) and Strokin and Reiser (2016 and 2017) in this model provide novel insights into the impact of iPLA2β mutations on cellular regulation and handling of Ca<sup>2+</sup>. However, these investigations were primarily focused in astrocytes and primary cultures of neurons and have provided minimal insight into two key features of PLAN and other NBIA disorders: the degeneration and iron accumulation inside of the BG. Characterization of these two hallmarks of NBIA in this model will support future research efforts using this model and provide novel insights into the disease processes in PLAN. Due to the prominent early-onset neurodegeneration seen in these mice, this investigation was conducted based on the hypothesis that significant degeneration of the BG occurs in B6.C3-Pla2g6<sup>m1J/CxRwb</sup> mice including a loss of dopaminergic function within the SN, which is directly related to the observed motor symptoms previously reported by (Strokin et al., 2012). Additionally, it was hypothesized that similar to PLAN patients these mice would
also show abnormal iron metabolism resulting in iron accumulation within the BG. To address these hypotheses this investigation assessed the previously reported motor symptoms and weight loss reported in the C3H/HeJ background of these mice in the novel B6.C3 background utilized in this study, measured the dopaminergic function based on TH expression in these mice, assessed the presence of neurodegeneration with the SNc and SNr and evaluated the SN and other midbrain areas for the presence of overt iron accumulation. Collectively, these efforts will help to address limitations in currently available models of NBIA by improving our understanding of B6.C3-Pla2g6\textsuperscript{m1J/CxRwb} mice as a model of PLAN. Furthermore, they will provide insight into the nature of degeneration within the basal ganglia in PLAN.

3.3. Methods

3.3.1. Mouse Model Selected

As noted above, the mice used for this study were a B6.C3 line containing the previously described Pla2g6\textsuperscript{m1J/CxRwb} mutation (Strokin et al., 2012). These mice were obtained from Jackson Laboratory (Stock No: 019062). This mutation featured an intracisternal A particle (IAP) insertion in the \textit{PLA2G6} gene. The insertion was inside of intron 1 upstream of the start codon (Strokin et al., 2012). This insertion results in a reduction of transcript levels in homozygous mice to 10% of wildtype levels (Strokin et al., 2012). All experimental animals used in this study were bred from heterozygous parents due to breeding limitations present in the homozygous mice. For details of breeding limitations in homozygous mice see Strokin et al. (2012).

Initially, this research was conducted based on the assertion that this was the sole mutation present in these mice. However, investigation in the mice noted an abnormal pattern of YFP fluorescence present endogenously within the mice. Communication and investigation in tandem with Jackson Laboratory staff revealed that these mice also contained the B6.C3-Tg (Thy1-YFP) transgene. As the presence of this additional fluorescent transgene was not accounted for in the initial setup of the investigations a reinterpretation of a selection of the imaging results was required. See Appendix B for
further details. THY1 is a cell surface glycoprotein which is highly expressed in nerves (Morris, 1985). It is a fairly ubiquitous surface component of neurons in the CNS. It does exhibit staining variability based on cell type, but it is known to be strongly expressed on GABAergic neurons (Pan, Brown, & Dudman, 2013).

3.3.2. Genotyping

All mice were genotyped for the Pla2g6<sup>m1J/CxRwb</sup> IAP insertion using the methods previously described by Strokin et al. (2012). This setup used a common forward primer and a separate reverse primer for wildtype and mutant animals (Table 3-1). PCR and gel electrophoresis using these primers (Eurofins Genomics) generates a single band at ~150 bp for mice homozygous for the IAP insertion, wildtype mice have a single band at ~280-300 bp. Heterozygous mice display both bands. All mice were ear punched for identification purposes at 30 days of age. Ear punches were stored at -20°C until utilization for genotyping. Each ear punch was digested with 50 mM NaOH (Caledon) for 30 min at 95°C. Digested ear punches were cooled briefly and neutralized with 1 M Tris-HCl (pH 5.5, Anachemia). All PCR primers were resuspended in a single mixture at 33 uM in ddH₂O (Promega). Diluted primers were mixed with digested DNA and PCR master mix (Promega). See Appendix C for master mix details.

PCR samples were electrophoresed for 70 min at 60 V using a 2% agarose gel and tris-acetic acid-EDTA running buffer. DNA was visualized under UV light using Sybr Safe™ (Sigma). Following completion samples were stored at -20°C. See Appendix C for PCR setup details and a reference gel.

Table 3-1. PCR primers utilized for the genotyping of B6.C3-Pla2g6<sup>m1J/CxRwb</sup> Mice.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2-Common Forward</td>
<td>TGTGGCATCTCTGGCTGGAAAC</td>
</tr>
<tr>
<td>PLA2-Mutant Reverse</td>
<td>GGCTCATGCGCAGATTATTT</td>
</tr>
<tr>
<td>PLA2-Wildtype Reverse</td>
<td>GGCTCATGCGCAGATTATTT</td>
</tr>
</tbody>
</table>

3.3.3. Study Design

Based on the previous findings of Strokin et al. (2012) of a rapid decline including severe weight loss and mortality in mice after 90 days of age, an experimental endpoint
of 90 days was selected for this study to limit morbidity and the potential for mortality in experimental mice. Wildtype (Pla2g6m1J/CxRwb−/−, PLA2+/+), heterozygous (Pla2g6m1J/CxRwb+/−, PLA2+/−) and homozygous (Pla2g6m1J/CxRwb+/+, PLA2+/+) mice were closely monitored over the course of the 90-day experimental period. Additional assessment of motor symptoms in mice was also performed at 30, 60, 80 and 90 days. At the end of the experimental period mice were sacrificed for evaluation of neurodegeneration within the SN based on fluorescent microscopy of tyrosine hydroxylase expression (TH) and THY1-YFP as well as PERLs stain evaluation of iron accumulation. A subgroup of 60-day old homozygous mice was also evaluated in this manner following their 60-day evaluation of motor symptoms.

3.3.4. Monitoring

Extensive monitoring of all mice was performed throughout the experimental period. This examination included the general appearance of the mice including signs of piloerection, grooming habits, hunched posture and overt signs of weight loss, the social interaction of the mice and any notable impairments in the general locomotion of the mice in the cages. Prior to 60 days of age mice were weighed once a week. After 60 days of age mice were weighed twice a week until the end of the study period.

3.3.5. Statistical Analysis and Graphical Presentation

Statistical analyses were conducted using R (R Core Team, 2016). Mice were compared by genotype using ANOVAs and post-hoc analyses with Tukey’s HSD test. Unless otherwise noted, p values of post hoc tests between genotypes are presented. Two-tailed students t-tests were used to compare 60-day old mice to 90-day old mice. Results were presented graphically using the R packages “ggplot2” (Wickham, 2009) and “gridExtra” (Auguie, 2016).

3.3.6. Motor Tests

Open Field Test

All mice were evaluated for spontaneous locomotion and general activity levels in a freshly prepared open field test arena (20 x 42 cm) at 30, 60, 80 and 90 days of age. See Appendix D for an overview of the open field test setup. The mice were recorded at 30
fps under red light. The mice were isolated for 3 min in the test arena prior to open field movement to allow them to acclimatize to the new environment and minimize stress due to handling and transfer. The mice were removed from isolation and allowed to freely explore the test arena for 5 min.

The recorded video MPEG file was converted to an AVI file using FFmpeg software. All video analysis was performed blinded to the genotype and sex of the mouse. Images were imported into ImageJ for analysis. Each video length was adjusted to remove the isolation period of the test. Videos were converted to an 8-bit format and a gaussian blur of 2.0 was applied. The position of the mouse in each frame of the video was converted to an X, Y coordinate and used to determine position, distance travelled and velocity over the course of the test. The total cage area was separated so that 50% of the area along the outside of the cage was established as the outer edge of the test arena, while the remaining 50% of the area at the center of the test area was the inner portion. This was used to determine the proportion of the test that each mouse spent in the outer edge of the test environment. Once processing was complete each test was unblinded. Distance travelled, velocity and the portion of the test spent on the inside of the cage were compared between groups.

**Wire Hang Test**

The mice were subjected to a brief wire-hang test at 60, 80 and 90 days of age. This behavioral test evaluates grip strength and motor coordination, and is described by Sango et al. (1996). A wire was suspended 40 cm above a 1-inch thick layer of soft bedding inside a 20 cm x 42 cm test arena. See Appendix E for an overview of the wire hang test setup and details. At 30 days of age the mice were acclimatized to being placed on the wire for 10 min. At 60, 80 and 90 days of age the mice were subjected to a measured wire hang test. Prior to each test the mouse weight was recorded. The tests were recorded at 30 fps under ambient light. For each test the mouse was placed inside the test arena for 30 seconds of acclimatization. Following acclimatization, the mouse suspended on the center of the wire, hanging by its forelimbs. A total test time of 3 min was used for each
of the mice. Each time the mouse fell or dropped off the wire it was allowed 5 seconds of recovery before being placed back on the wire. In the event that a mouse remained on the wire at the end of the 3-minute test period up to 1 minute of additional time was allotted to determine the maximal ability of the mice and to avoid biasing the results with falsely truncated hang times.

Video analysis of wire hang tests was performed blinded to the genotype and sex of the mice. The total test time, the amount of the test spent on the wire, the maximum impulse (weight normalized time without falling or dropping off the wire), total impulse over the entire test, number of drops, number of falls, whether the mouse was able to pull itself up and balance on the wire and the number of times the mouse was able to move laterally along the wire and reach either side of the test arena was recorded for each test. Each of the falls was analyzed to determine if the mouse fell off of the wire due to a lack of motor strength or if they fell for other reasons (failed to grasp the wire, or if they fell off while balancing). See Appendix E for reference images of all noted events.

The fall data was used to construct a falls test score in the style of a survival curve. Each fall that occurred during the test resulted in a one-point deduction from an initial score of 10 at the corresponding time of the test. This was tracked over the entire portion of the test. Due to differences in the total test time and time the mice were on the wire between tests this time data was converted to percentage of the total test time. A Falls/Reaches score was also calculated for each test. Starting from an initial score of 10 each mouse had 1 point added to the score each time they reached the side of the test chamber on the wire and a deduction of 1 each time they fell off of the wire. Once data analysis was completed all mice were unblinded. Groups were compared by their total time on the wire, maximum impulse, total impulse, number of falls and total number of reaches.

3.3.7. Cardiac Perfusion

Once mice reached the end of the study period they were euthanized using a combination of isoflurane (Fresnius Kabi) and cardiac perfusion with PBS (Sigma). Heparin
(Sigma) treated PBS (10 IU/mL, pH 7.6) was perfused intracardially. PBS perfusion was followed by perfusion with 4% paraformaldehyde (PFA, Electron Microscopy Sciences) diluted in heparin treated PBS (10 IU/mL, pH 7.6) for fixation. Following fixation perfusion with 20 mL of 5% sucrose (Anachemia) was performed to ensure that residual PFA was rinsed from the brain; lowering potential autofluorescence from the PFA. Finally, the brain was removed and stored for 2-3 days at 4°C in 30% sucrose.

3.3.8. *Slicing Protocol*

Following 30% sucrose treatment, each brain was trimmed ventrally in the frontal plane, mounted in OCT medium (Tissue Plus, Fisher Scientific) and rapidly frozen on dry ice. The mounted brains were sliced at 30 to 40 μM thick coronal sections using a Leica CM1850 Cryostat. Slices were collected from the substantia nigra (-2.8 to -3.52 Bregma) and mounted on coated slides (Superfrost Plus Gold, Fisher Scientific). All slides were stored at -20°C until staining and imaging.

3.3.9. *Tyrosine Hydroxylase Staining*

Slides were removed from -20°C storage and thawed. Each slide was rinsed briefly with PBS (unless otherwise noted PBS was utilized at pH 7.6). Each slide was washed twice for 10 min via incubation with PBS. Following incubation, the slides were incubated with 10% donkey serum (Jackson ImmunoResearch) in PBS for 30 min at room temperature. After incubation the 10% donkey serum was removed from the slides. Rabbit anti-tyrosine hydroxylase polyclonal antibody (Pel-Freez) was used as the 1° antibody for the staining of TH. The 1° antibodies were thawed and spun for 5 min at 14000g. 1° antibodies were diluted 1:200 in 3% donkey serum in PBS. Diluted 1° antibody was added to each slide. Slides were incubated overnight (~16 hours) at 4°C. Each slide was washed 3 times for 5 min with PBS to remove the primary antibody. The 2° antibody; Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), was diluted 1:200 in 3% donkey serum and added to each slide. Slides were incubated overnight (~16 hours) at 4°C. After overnight incubation, each slide was rinsed 3 times for 10 min with PBS. Slides were dried briefly at 37°C. Immunomount (Shandon, Fisher Scientific) previously adjusted to a pH of 8.4 with
1M NaOH) was added to each slide and a coverslip was added. The immunomount was dried at room temperature for 30 min prior to imaging.

3.3.10. Fluorescent Imaging

All fluorescent imaging of coronal sections was performed using a Nikon C1si spectral confocal system attached to a Nikon Eclipse Ti-E inverted microscope. A series of 5 images was collected for each fluorescence probe for each side of the coronal section of the SN and surrounding area (See Appendix F for an overview of imaging setup and locations in the SNc and SNr). These images were taken from Bregma -2.7 to Bregma – 3.6. Using a Nikon 10x CFI PL Fluor objective (NA = 0.13, 13 mm working distance) 1273 µm x 1273 µm images were collected of I) the VTA as well as II) the entirety of the SN. Using a Nikon 20x CFI PLAN Apochromat objective (NA = 0.75, 1.0 mm working distance) 636.5 µm x 636.5 µm images were collected of I) the VTA and medial SN, II) the remainder of the medial SN and the central SN and III) the lateral SN. Excitation of Cy3 was performed using a 561 nm diode pumped solid state laser at 3% of maximum transmission for the 10x objective and 1% transmission for the 20x objective. Excitation of THY1-YFP was performed using a 488 nm laser line of a 40 mW Argon laser at 1% transmission for the 10x objective and 0.5% transmission for the 20x objective. As detailed in (Renda & Nashmi, 2012) the spectral confocal system was used to collect a lambda stack of images in 32 5 nM increments across the detection wavelength range of 160 nm. A Bregma location was assigned to all slides based on the size and shape of the SNc and its accompanying TH positive neurons, the shape of the VTA as well as the shape of the ventricles. A single set of optimized settings were used at each magnification to ensure comparability of fluorescent intensity for all 20x images which were used for quantification. Standardized settings were not utilized for 10x images which were used solely for localization and as such the relative TH intensity of the 10x images is not directly comparable and is only used to represent the trend quantified in the 20x images (This applies to Figure 3-3A, D, G and J). Settings were identical for all sections during imaging and the laser power was checked with a power meter prior to each day's experiment to ensure consistency. Using linear unmixing, which is detailed in full by Dickinson, Bearman,
Tille, Lansford, & Fraser (2001), the lambda stack was deconvolved into the respective fluorescent signals as well as autofluorescence based on the previously recorded spectrums for each fluorescent probe as well as background autofluorescence. A representation of the location utilized for fluorescent imaging is also provided (Figure 3-1).

![Figure 3-1](image-url)  
**Figure 3-1.** Representative image of a coronal section taken from a mouse brain which encompasses the substantia nigra (SN) (A). The areas imaged in this study which encompasses the substantia nigra pars compacta (SNC), substantia nigra pars reticulata (SNR) and a portion of the ventral tegmental area (VTA) are highlighted (Red Box). The location of imaging along the length of the mouse brain in the sagittal plane is highlighted (B). This figure is modified from the original in the work of Franklin and Paxinos (2008).

### 3.3.11. TH Image Analysis

All image assessment was preformed blinded to mouse age and genotype. Using ImageJ both the SNc and SNr were outlined based on the extent of TH positive neurons and fluorescence as well as the known shape of the SNc and SNr at the assigned Bregma location. The SNc was outlined in 3 images which captured the medial, central and lateral sections of the SNc respectively at 20x magnification. The SNr was outlined in 2 images which captured the medial and lateral portions of the SNr at 20x magnification. The total area and mean intensity of each of these regions was recorded. A secondary assessment
was done of the outlined areas and all visible neurons were counted. Neuron counts and area measurements were used to determine the density (neurons/pixel) of neurons. Following this analysis, the images were unblinded and were pooled based on genotype, brain region and Bregma location. Once data analysis was completed all mice were unblinded and statistical analyses were performed to compare groups by area, mean TH intensity, neuron counts, neuron density and mean area.

3.3.12. **THY1-YFP Image Analysis**

All image assessment was preformed blinded. Any spheroid noted with the SNr were counted using a standardized set of morphological characteristics. Following image analysis all images were unblinded. Statistical analyses were performed comparing groups by spheroids counts.

3.3.13. **PERLS Stain with Diaminobenzidine Amplification**

All steps in the outlined protocol were performed at room temperature and protected from light unless otherwise noted. Following primary imaging for TH, each slide was stored at 4°C until a follow-up PERLS/Diaminobenzidine (DAB) stain was performed. Slides were soaked in PBS (pH 7.4) for 2 to 5 days with gentle agitation to solubilize immunomount. Once the immunomount was solubilized each coverslip was carefully removed. Slides were washed 3 times for 2 min in PBS (pH 7.4) following the removal of the coverslips. Each slide was incubated with PERLS reagent (5% Potassium Ferrocyanide (Sigma) and 5% HCl in ddH₂O) for 60 min at room temperature. After exposure to PERLS reagent, each slide was gently rinsed with ddH₂O and washed 3 times for 2 min with ddH₂O. Each slide was then treated with a 0.075% DAB (Sigma) solution in PBS for 30 min to amplify the PERLS stain. After 30 min an equal volume of 1% H₂O₂ (Caledon) was mixed with the DAB solution on each slide and incubated for an additional 30 min. Each slide was gently rinsed with ddH₂O and washed 3 times for 2 min with ddH₂O to remove any residual stain. Slides were briefly dried in an incubator at 37°C and mounted using 90% glycerol (ACP Chemicals) in PBS (pH 7.6).
PERLS/DAB Imaging

Stained slides were imaged on an Olympus BX51-FL microscope using a X-Cite® 120Q lamp for Cy3 excitation. Imaging was performed in duplicate for each slide. The first set of images was taken using the Cy3 filter to visualize previous staining with TH specific antibodies. A second image was taken under bright field illumination to visualize the PERLS/DAB stain. 3480 µm x 2610 µm images of the midbrain were collected using a Nikon UPLFLN 4x objective (NA = 0.13, 17 mm working distance). Cy3 fluorescence of TH positive neurons was used to locate the SN using a Nikon PLN 10x objective (0.25 NA, 10.6 mm working distance). 1370 um x 1028 uM bright field images of the SN as well as the VTA were collected using a PLN 10x objective (0.25 NA, 10.6 mm working distance). Standardized illumination was used for each bright field image to ensure comparability of slides.

PERLS/DAB Image Processing

All PERLs/DAB Images were blinded of any identifying information prior to the initiation of analysis. Image analysis was performed using ImageJ software. Each RGB image was converted to an 8-bit image and the intensity values were inverted (so areas with darker PERLS/DAB stain had a higher intensity). The total area of the midbrain was outlined and the mean intensity of the PERLS/DAB stain was calculated. Cy3 fluorescence was used to outline the SNC and SNR. The mean intensities of the SNC, SNR and the full SN were calculated. Following the completion of this analysis all slides were unblinded. The mean intensity and area were compared statistically between groups.

3.3.14. Secondary Data Analyses

The severity of motor symptoms in the mice as measured by their Falls/Reaches test score was compared to the mean intensity of TH fluorescence. Pearson’s correlations between the mean TH intensity in each brain region and the falls reaches score for each mouse were calculated. Additional correlations were calculated between the severity of iron accumulation, based on the PERLS/DAB stain intensity and the Falls/Reaches score of each mouse.
3.4. Results

3.4.1. Monitoring

No notable differences were seen during monitoring in the Pla2g6\textsuperscript{m1J/CxRwb +/+} mice (homozygous, PLA2\textsuperscript{-/-}) compared to the Pla2g6\textsuperscript{m1J/CxRwb +/-} mice (heterozygous, PLA2\textsuperscript{+/-}) or Pla2g6\textsuperscript{m1J/CxRwb -/-} mice (wildtype, PLA2\textsuperscript{+/+}) prior to 60 days of age. A minor tremor was discernable in the homozygous mice between 65-70 days of age. This tremor became more pronounced over time and was overtly apparent by 90 days of age. No differences in the appearance of the mice were observed. Similarly, grooming habits and posture were normal. No adverse social interactions were seen in the mice, notably the heterozygous and wildtype mice did not isolate or attack their homozygous littermates. The general locomotion of the homozygous mice in the cage was slightly slower and more hesitant at 90 days. When prodded the mice resumed normal locomotion.

3.4.2. Mouse Weights

No notable difference was seen in the weights of the homozygous mice in the 90-day study period (Figure 3-2). This trend was also evident when looking at male mice by genotype (Supplementary Figure 0-5B) and female mice by genotype (Supplementary Figure 0-5C). A slight non-significance decline in weight was seen in the homozygous mice at the end of the study period. In a single female homozygous mouse degeneration was more rapid; a >20% weight loss was observed at 90 days.
3.4.3. Wire Hang Test

A significant decline in motor strength and coordination was seen in the homozygous mice during the study period as measured by a wire hang test. When the falls were plotted as a Falls Test Score in comparison to the time that they occurred during the test; as a percent, all mice showed a strong ability to remain on the wire at 60 days of age (Figure 3-3A). A rise in the number of falls over the course of the study period was seen in the homozygous mice (Figure 3-3B). By 90 days the homozygous mice showed a distinct separation from the wildtype and heterozygous mice, which showed no change in motor ability (Figure 3-3C). Notably at 90 days of age, the homozygous mice showed a stronger motor ability during the initial portion of the test, with the majority of the decline in the Falls Test Score occurring during the second half of the test (Figure 3-3, B and C).

At 60, 80 and 90 days of age homozygous mice had less lateral movement on the test wire (Supplementary Figure 0-6A). This was accompanied by a significantly higher number of falls off of the wire at 90 days than the wildtype mice, $p = 0.001$, and heterozygous mice $p = 0.007$ (Supplementary Figure 0-6B). A decline in the Falls/Reaches test score was seen in the homozygous mice at 80 days and a significant decline was seen at 90 days when compared to the wildtype, $p = 0.004$, and heterozygous mice, $p = 0.04$ (Supplementary Figure 0-6C). The falls which occurred in the wildtype and heterozygous
mice were predominantly due to the mice losing their balance on top of the wire, while the falls which occurred in the homozygous mice were due to a loss of motor strength (Data not shown). By 90 days of age all of the homozygous mice, excluding a single more slowly progressing mouse, had lost the ability able to pull themselves up and balance on top of the wire, while almost all of the heterozygous, \( p = 0.006 \), and all of the wildtype mice were able to, \( p < 0.001 \) (Supplementary Figure 0-6D). This decline in motor ability was also seen in the weight adjusted time the mice were able to remain on the wire (impulse). Both the total (Supplementary Figure 0-7A) and the maximum impulse (Supplementary Figure 0-7B) were significantly reduced in the homozygous mice compared to the wildtype, \( p = 0.02 \) and \( p = 0.036 \) respectively, and heterozygous mice, \( p = 0.05 \) and \( p = 0.046 \) respectively.

**Figure 3-3.** Mouse average falls scores over test time, as a percent, during wire hang tests for wildtype (PLA2\(^{+/+}\), \( n = 7 \ (6F/1M) \)), heterozygous (PLA2\(^{+/-}\), \( n = 5 \ (3F/2M) \)) and homozygous (PLA2\(^{-/-}\), \( n = 6 \ (3F/3M) \)) mice. At 60, 80 and 90 days of age mice were placed on a suspended wire for 3 min and test behaviour was recorded. Each fall off of the wire resulted in a 1-point deduction from a starting total of 10 at the
corresponding test time. Tests times were converted to a percent of the total test time to account for variability between test durations. At 60 days of age no notable difference was seen in the mice by genotype (A). Homozygous mice showed a steady decline in Falls Score from 60 days to 90 days (B). This decline contrasted with the steady scores of heterozygous and wildtype mice at 90 days (C). Bands represent SE.

3.4.4. Open Field Test

No notable difference was seen in the open field locomotion of the homozygous mice during the study period; the total distance moved by the mice (Supplementary Figure 0-8A), the average velocity of the mice (Supplementary Figure 0-8B) and percent of the test time actively moving (Supplementary Figure 0-8C) were not significantly different by genotype. A trend of an increasing portion of the test being spent in the interior portion of the test arena by the mice was seen in all three genotypes (Supplementary Figure 0-8D).

3.4.5. Tyrosine Hydroxylase Fluorescent Imaging

A decrease in the mean intensity of TH staining was seen in the homozygous mice at 90 days or age (Figure 3-4, J-L) in comparison to the wildtype (Figure 3-4, A-C), heterozygous (Figure 3-4, D-F) and 60-day old homozygous mice (Figure 3-4, G-I). This was quantified in the SNc which is the uppermost portion of the SN which is rich in dopaminergic neurons as well as the SNr which is the lower portion of the SN, which is largely absent of neuronal cell bodies, but does have dopaminergic projections. When compared at 90 days by region this decrease in the mean intensity of TH staining in the homozygous mice was significant decreased compared to the wildtype and heterozygous mice in the medial SNc, p = 0.008 and p = 0.008, lateral SNc, p = 0.024 and p = 0.05, and total SNc, p<0.001 and p<0.001 (Figure 3-5A). The central SNc was significantly decreased in the heterozygous, p = 0.005, but not the wildtype mice (Figure 3-5A). No significant change was noted in the mean TH intensity in the SNr by genotype (Figure 3-5B). When the 90-day old homozygous mice were compared to the 60-day old homozygous mice, a significant reduction of mean TH intensity was seen in both the SNc (Figure 3-6A) and the SNr (Figure 3-6B). A significant reduction in mean TH intensity was seen in the Medial SNc, p<0.001, Lateral SNc, p = 0.025, and total SNc, p = 0.01, in homozygous mice at 90 days compared to homozygous mice at 60 days. The mean TH intensity in the central SNc was
reduced, but not significantly so. Additionally, a slight reduction in the mean TH intensity was observed in the Medial SNr and Lateral SNr (Figure 3-6B).
Figure 3-4. Representative fluorescent images of the substantia nigra in mice visualized using Cy3 targeted to anti-tyrosine hydroxylase antibodies in wildtype (PLA2^{++}), heterozygous (PLA2^{+/-}) and homozygous (PLA2^{-/-}) mice. Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 10x CFI PL Fluor objective (A, D, G, and J) and a Nikon 20x CFI PLAN Apochromat objective (B+C, E+F, H+I, and K+L). Images were processed using linear unmixing using previously captured...
Cy3 and SN autofluorescent spectrums. Homozygous mice at 90 days of age (J-L) show a marked reduction in TH intensity compared to wildtype mice at 90 days of age (A-C), heterozygous mice at 90 days of age (D-F) and homozygous mice at 60 days of age (G-I). SNc and SNr were traced in blinded images based on the extent of TH positive staining with reference to know SN morphology for the assigned Bregma location. The overview slides at 10x magnification were not used for quantification.

**Figure 3-5.** Mean intensity of Cy3 targeted to anti-tyrosine hydroxylase antibodies in the substantia nigra (SN) of 90-day old wildtype (PLA2+/+, n = 40 slides from 5 mice (4F/1M)), 90-day old heterozygous (PLA2+/-, n = 40 slides from 5 mice (3F/2M)) and 90-day homozygous mice (PLA2-/-, n = 50 slides from 6 mice (3F/3M)). Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing with previously captured Cy3 and SN autofluorescent spectrums and mean intensity was measured in each region using ImageJ. A reduction in tyrosine hydroxylase (TH) intensity was seen across regions of the SN pars compacta (SNc) in homozygous mice at 90 days compared to heterozygous and wildtype mice (A: Medial, Central and Lateral). The average of all three regions was significantly lower than wildtype $p = 0.019$, * and heterozygous mice $p<0.001$, ***, (ANOVA with Tukey’s HSD test). No significant difference in TH intensity was seen in the SN pars reticulata (SNr, B). Error bars represent SE.
Figure 3-6. Mean intensity of Cy3 targeted to anti-tyrosine hydroxylase antibodies in the substantia nigra (SN) of 60-day old homozygous (PLA2\textsuperscript{+/+}, n = 30 slides from 3 mice (2F/1M)) and 90-day old homozygous mice (PLA2\textsuperscript{--}, n = 50 slides from 6 mice (3F/3M)). Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing with previously captured Cy3 and SN autofluorescent spectrums and mean intensity was measured in each region using ImageJ. A reduction in tyrosine hydroxylase (TH) intensity was seen across regions of the SN pars compacta (SNc, A: Medial, Central and Lateral) in homozygous mice at 90 days of ages compared to homozygous mice at 60 days of age. The average of all three regions was significantly lower at 90 days, \( p = 0.01 \), *, (2-tailed, unpaired, student’s t-test). A reduction in mean TH intensity was seen in the SN pars reticulata (SNr, B) across both the medial and lateral portion. The average of all both regions was significantly lower at 90 days, \( p = 0.007 \), **. (2-tailed, unpaired, student’s t-test). Error bars represent SE.

An interesting trend was seen in the number of TH positive neurons inside of the SNr between the 60-day old and 90-day old homozygous mice. There was a notable increase in the number of TH positive neurons inside of the lateral portion of the SNr in 60 day old mice (Supplementary Figure 0-9, A-C) in comparison to the lateral portion of the SNr in 90 day old homozygous mice (Supplementary Figure 0-9, D-F). A significant reduction the number of TH positive neurons was seen in the lateral portion of the SNr, \( p<0.001 \) (Supplementary Figure 0-10A). This reduction was concentrated at Bregma locations < -3.2 (Supplementary Figure 0-10B).
3.4.6.  THY-1 Fluorescent Imaging

THY1-YFP fluorescent imaging revealed the formation of small inclusion-like spheroids (hereafter referred to as spheroids) predominantly in the lateral portion of the SNr in homozygous mice. These spheroids were visible at 60 days of age (Figure 3-8, G-I) and became more pronounced by 90 days of age (Figure 3-8, J-K). No signs of spheroids were seen amongst the neurons in the wildtype (Figure 3-8, A-C) or heterozygous mice in the midbrain (Data not shown) or in the SNr (Figure 3-8, D-F). A significant increase in the number of spheroids was observed in homozygous mice in the medial and lateral SNr in comparison to heterozygous and wildtype mice who were absent for spheroids, p<0.001 and p<0.001 (Figure 3-9A). The formation of spheroids was observed to increase with age. 90-day old homozygous mice had a significant increase in the number of spheroids in both the medial and lateral SNr in comparison to 60-day old homozygous mice, p = 0.024 and p = 0.026 (Figure 3-9B). Less concentrated, widespread spheroid formation was seen in homozygous mice at 90 days of age throughout the midbrain (Figure 3-7, A and B), but not in the cerebrum (Figure 3-7C).

![Figure 3-7](image.png)

Figure 3-7. Representative fluorescent images of the midbrain and cerebrum visualized using THY1-YFP in 90-day old homozygous (PLA2-/-) mice. Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing using previously captured YFP and SN autofluorescent spectrums. Homozygous mice at 90 days of age show a marked increase in the formation of small inclusion-like bodies throughout the midbrain including the red nucleus (A) as well as the central region of midbrain which contains portions of the ventral tegmental area and red nuclei (B). A sparsity of inclusion formation was seen inside of the cerebrum (C).
Figure 3-8. Representative fluorescent images of the substantia nigra visualized using THY1-YFP in wildtype (PLA2^{+/+}), heterozygous (PLA2^{+/-}) and homozygous (PLA2^{-/-}) mice. Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 10x CFI PL Fluor objective (A, D, G, and J) and a Nikon 20x CFI PLAN Apochromat objective (B+C, E+F, H+I, and K+L). Images were processed using linear unmixing using previously captured YFP and SN autofluorescent spectrums. Homozygous mice at 90 days of age (J-L) show a marked increase in the formation of small inclusion-like bodies (grey circles) amongst the neurons (white squares) of the substantia nigra pars reticulata (SNr) compared to wildtype mice at 90 days of age (A-C) and heterozygous mice at 90 days of age (D-F) which are absent for inclusion formation. This trend was also noted to a lesser degree in homozygous mice at 60 days of age (G-I).
Figure 3-9. Mouse substantia nigra pars reticulata (SNr) inclusion counts of wildtype (PLA2+/+, n = 19 slides from 5 mice (4F/1M)), heterozygous (PLA2+/−, n = 18 slides from 5 mice (3F/2M)), 60 days homozygous (PLA2−/−, n = 15 slides from 3 mice (2F/1M)) and 90-day homozygous mice (PLA2−/−, n = 31 slides from 6 mice (3F/3M)). Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing using previously captured YFP and SN autofluorescent spectrums and a count was taken in each region using ImageJ. The formation of spheroids was seen in the medial and lateral SNr of 90-day old homozygous mice (A), but not in heterozygous, p<0.001, *** (2-tailed, unpaired, student’s t-test), or wildtype mice, p<0.001, *** (2-tailed, unpaired, student’s t-test). A significant increase in the formation of spheroids was seen between 60 days and 90 days of age in the homozygous mice in both the medial and lateral SNr (B), p = 0.024 and p = 0.026, * (2-tailed, unpaired, student’s t-test). Error bars represent SE.

The formation of these spheroids and the presence of the likely GABAergic neurons within the SNr did not overlap with TH staining (Data Not Shown). This comparison was performed visually in coronal slices which contained both endogenous THY1-YFP expression as well as TH antibody labelling.

3.4.7. PERLS/DAB Staining of Iron Levels

A marked increase in the intensity of PERLs stain with DAB intensification was seen in the SN of homozygous mice at 60 days (Figure 3-10G) and 90 days of age (Figure 3-10J) compared to wildtype (Figure 3-10A) and heterozygous mice (Figure 3-10D). TH fluorescent staining was maintained following the PERLS/DAB staining process (Figure 3-10B, E, H and K). This fluorescence was used to locate the SN in PERLS/DAB stained slides (Figure 3-10C, F, I and L) Some autofluorescence from the PERLS/DAB stain was seen using the Cy3 filter (Minor autofluorescence is seen in Figure 3-10E).
Figure 3-10. Representative PERLS stained images of the substantia nigra with diaminobenzidine (DAB) intensification of the in wildtype (PLA2<sup>+/-</sup>), heterozygous (PLA2<sup>+/</sup>) and homozygous (PLA2<sup>-/-</sup>) mice at 90 days of age as well as homozygous mice (PLA2<sup>-/-</sup>) at 60 days of age. Bright field images were taken on an Olympus BX51-FL microscope using a PLN 10x objective (A, D, G and J). The SN was located using Cy3 targeted to anti-tyrosine hydroxylase antibodies visualized with an X-Cite® 120Q lamp and Cy3 filter (B, E, H and K). This was overlaid on top of bright field images to determine the location of the SN (C, F, I and L). Homozygous mice at 90 days of age (J-L) show a marked increase in the intensity of PERLS/DAB stain of the SN compared to wildtype mice at 90 days of age (A-C) and heterozygous mice at 90 days of age (D-F). This trend was also noted to a lesser degree in homozygous mice at 60 days of age (G-I).

The mean intensity of PERLS/DAB staining was increased in the SNc, SNr and SN, but not in the midbrain in homozygous mice at 90 days compared to wildtype and heterozygous mice.

Figure 3-11A). This increase was significant in the heterozygous mice in the SNc, \( p = 0.021 \), SNr, \( p = 0.031 \), and full SN, \( p = 0.025 \), and approaching significance in the wildtype mice in the SNc, \( p = 0.14 \), SNr, \( p = 0.17 \), and full SN, \( p = 0.14 \). This significance increased when the unaffected mice were pooled.
Figure 3-11B), SNc, $p = 0.009$, SNr, $p = 0.008$, full SN $p = 0.007$. Only a slight increase was seen between the 60-day old homozygous mice and 90-day old homozygous mice in PERLs/DAB stain intensity, which was not significant (Figure 3-11C).
3.4.8. **Secondary Analyses**

A significant correlation was seen between decreased TH expression and decreased motor strength and coordination on the wire hang test. The mean intensity of TH and the Falls/Reaches test score on the wire hang test were strongly correlated at 90 days of age (Figure 3-12 and Supplementary Figure 0-11). This correlation was significant in the medial SNc (Figure 3-12A), \( p<0.001 \) \( R = 0.92 \), central SNc (Figure 3-12B), \( p = 0.001 \) \( R = 0.75 \), lateral SNc (Figure 3-12C), \( p = 0.007 \) \( R = 0.66 \), and the average of the full SNc (Figure 3-12D), \( p<0.001 \) \( R = 0.87 \). A similar trend was noted in the medial SNr (Supplementary Figure 0-11A), \( p = 0.03 \) \( R = 0.57 \), lateral SNr (Supplementary Figure 0-11B), \( p = 0.04 \) \( R = 0.53 \) and the average of the full SNr (Supplementary Figure 0-11C), \( p = 0.02 \) \( R = 0.57 \). Notably, a single homozygous mouse which did not strongly show motor symptoms at 90 days of age had a markedly increased intensity of TH staining in all regions compared to the other homozygous mice.
Figure 3.12. Correlations between the mean intensity of Cy3 targeted to anti-tyrosine hydroxylase antibodies in the substantia Nigra pars compacta (SNc) and the final Falls/Reaches score on the wire hang test of 90-day old wildtype mice (PLA2\(^{++}\), n = 5 (4F/1M)), heterozygous mice (PLA2\(^{+/-}\), n = 4 mice (3F/1M)), and homozygous mice (PLA2\(^{-/-}\), n = 6 mice (3F/3M)). A significant correlation was seen between the test score and the mean intensity in the substantia Nigra pars compacta (SNc) medial, \(p<0.001\), SNc central, \(p = 0.001\), SNc lateral, \(p = 0.007\), and the full SNc, \(p<0.001\) (Pearson’s Correlation).

3.5. Discussion

The purpose of this study was to investigate the previously reported (Strokin & Reiser, 2016, 2017; Strokin et al., 2012) Pla2g6\(^{m1J/CxRwb}\) mutation, which was reported to cause prominent early-onset neurodegenerative motor symptoms in a novel B6.C3 background. Two additional hallmarks of NBIA: iron accumulation and degeneration within the BG, which have also been poorly covered by existing models were also investigated. This investigation showed that a severe motor deficit consistent with the findings previously reported by (Strokin et al., 2012) in C3H/HeJ- Pla2g6\(^{m1J/CxRwb}\) mice is
also present in B6.C3 Pla2g6<sup>m1J/CxRwb</sup> mice. This was accompanied by degeneration and inclusion formation with the SNr as well as the loss of TH activity in the dopaminergic neurons of the SNc. Furthermore, we provide novel evidence of iron accumulation within the SN of B6.C3 Pla2g6<sup>m1J/CxRwb</sup> mice.

The first overtly visible signs of neurodegeneration within these mice was the emergence of a minor tremor between 65 to 70 days of age. Mice weight remained stable throughout the study period with no difference seen throughout the study period, this finding was consistent in both males and females. The timing of these events contrasts slightly to the work of Strokin et al. (2012), which saw the emergence of a tremor by 50 days and separation in weight beginning at 85 days in homozygous mice. These differences may be due to the different strains used in the two studies as well as due to the use of specialized soft paper bedding and low hanging food hoppers to support the mice in this study. By the end of the study period the homozygous mice were no longer gaining weight and in a single case a female mouse experience more rapid degeneration which included a prominent weight loss (>20% by 90 days). Based on these observations it is likely that the weight loss noted by Strokin et al. (2012) still occurs in the B6.C2 mice, just at a delayed time point. No significant morbidity and no mortality occurred during the 90-day trial period in the homozygous mice. We cannot confirm if the mortality reported by Strokin et al. (2012) also occurs in these mice. However, based on the similarity in disease progression observed in the first 90 days between the B6.C3 and C3H/HeJ mice we would hypothesize that significant mortality would occur in these mice with continued disease progression.

By 90 days of age a deficit in iPLA2β resulted in a pronounced tremor that was visible in all of the homozygous mice. This tremor did not significantly impact the general locomotion and exploratory behaviour of the homozygous mice in the open field test. Acclimatization to the test was observed in all of the mice tested. Early tests in the unfamiliar environment saw all of mice tested, regardless of genotype, spending the majority of the test sheltering near the edges. As the mice underwent repeated tests at 30, 60, 80 and 90 days of age they became acclimatized to the test environment and were
comfortable spending a larger portion of time in the center of the arena. This acclimatization could easily influence the results of mice undergoing repeated open field tests and should be accounted for in studies utilizing testing of mice in this fashion.

A severe and progressive deficit was seen in the homozygous mice on the wire hang test. At 60 days of age no motor deficit was detectable in the homozygous mice, but they rapidly progressed and by 90 days of age many homozygous mice struggled to hold themselves on the wire for an extended period of time and frequently fell off the wire during the test. This deficit was particularly pronounced in the second half of the test period, suggesting that the muscular endurance of the iPLA2β mice may also be affected. The homozygous mice also lacked the strength to pull themselves upright on the wire. This impairment on the wire hang test parallels the findings of Strokin et al. (2012) and confirms that a deficiency in iPLA2β leads to a severe motor deficit in these mice.

In this study, the wire hang test was a more sensitive test to detect motor deficits in Pla2g6m1J/CxRwb mice than open field tests of general locomotion. It is likely that the homozygous mice have a more familiarity with general locomotion within their cages and can preserve this ability more readily then motor strength and coordination; which they would not be able to utilize as easily in their cages. This would result in deficits in motor strength and coordination being detectable earlier than deficits in locomotion. This was also seen in an investigation by Shinzawa et al. (2008), which demonstrated early motor deficits in a different strain of iPLA2β knockout mice which limited their ability to hang suspended on a wire hang test. These deficits were more pronounced and detected earlier in the disease progression than deficits in movement and gait in the mice. This confirms our findings and provides evidence that wire hang tests represent an assessment method for motor deficits in iPLA2β mice that is more sensitive and able to detect deficits earlier than alternate methods, such as assessments of gait or open field locomotion.

Signs of degeneration inside the BG were confirmed accompanying the motor symptoms observed in the mice. A significant reduction in the expression of TH was shown in the SNc of the homozygous mice at 90 days of age; representative of a reduction
in dopaminergic function in the SNc. To our knowledge this is the first report of loss of dopaminergic function in a model of NBIA. The loss of dopaminergic function in the SNc impairs the normal function of the SNc, which is the regulation of the activity of the striatum and through it ultimately the thalamus (Sealfon and Olanow 2000). As has been robustly proven in PD (Dauer & Przedborski, 2003), the ultimate consequences of a loss of dopaminergic function in the SNc is the presentation of severe and progressive motor symptoms. Therefore, this novel finding of a reduction in TH in the SNc confirms that the motor deficit seen in these mice may be at least partially due to a loss of dopaminergic function in the SNc. This is not an unexpected finding as diminished dopaminergic activity has frequently been noted in models of PD (Devos et al., 2014; Dauer & Przedborski, 2003), as well as studies of patients with NBIA (Agarwal et al., 2012) and PD (Jankovic, 2008), but to our knowledge this is the first report of loss of dopaminergic function in a model of NBIA. Interestingly, there was no significant loss of dopaminergic positive neurons with the SNc. Instead, the overall TH expression levels of the region were decreased. Based on other studies in PD (Damier, Hirsch, Agid, & Graybiel, 1999; Dauer & Przedborski, 2003; Jankovic, 2008) which show a widespread loss of dopaminergic neurons in the SNc during disease progression it is likely that neuronal loss occurs in later disease stages in these mice. Earlier disease stages may be characterized by a reduction in dopaminergic function which precedes the loss of these neurons. Further evidence for this loss of dopaminergic activity in the SNc underlying the motor symptoms seen in the mice was seen in the investigation of the 60-day old homozygous mice, which showed no reduction in TH activity. These mice displayed no overt motor symptoms, and showed no deficit compared to the wildtype and heterozygous mice on the wire hang test. Additionally, a direct relationship between a reduction in TH levels and worse performance on the wire hang test was shown in the mice. Collectively, these findings provide strong evidence that reduced TH activity leads to a motor deficit in the B6.C3 Pla2g6<sup>m1J/CxRwb</sup> mice.

The presence of inclusions, or abnormal spheroid structures, which form axonally in association with swollen, dysfunctional mitochondria is a commonly seen hallmark in
models of PLAN (Beck et al., 2011; Kinghorn et al., 2015; Shinzawa et al., 2008) as well as patients with PLAN (Gregory et al., 2009; Gregory & Hayflick, 2013; Hogarth, 2015). Here we demonstrate the formation of abnormal spheroids predominantly with the SNr of mice deficient in iPLA2β. Dispersed formation of spheroids was also seen throughout the midbrain of homozygous mice. These spheroids were visualized using THY1-YFP. THY1 is strongly expressed as a surface glycoprotein on many types of neurons, including GABAergic neurons. It has been used in previous studies as a marker to locate GABAergic neurons in the SNr (Pan et al., 2013), which are the predominant neuronal cell type in the SNr (Alexander, DeLong, & Strick, 1986; Alexander & Crutcher, 1990; Lee, 2007). Based on these findings, the strongly THY1 positive cells noted within the SNr using THY1-YFP are likely GABAergic neurons. At 60 days the formation of abnormal spheroid is seen amongst the THY1-YFP positive cells within the SNr in homozygous mice. These spheroids increase in prevalence with age and numerous and dense spheroid formation is observed by 90 days of age. The visible staining of these spheroids, which are characteristic of PLAN, with THY1-YFP may be due to the accumulation of THY1 positive membranes within these spheroids or due to produced THY1-YFP being halted in its axonal transport in these neurons, causing it to accumulate. Notably, Strokin et al. (2012) noted the widespread formation of ubiquitin positive spheroids in the brains of the C3H/HeJ-Pla2g6^{m1J/Rwb} mice. The dispersed formation of these ubiquitin positive spheroids described by Strokin et al. (2012) matches the formation of THY1-YFP positive spheroids throughout the midbrain of homozygous mice, providing evidence that this spheroids formation is in conjunction with the accumulation of ubiquinated proteins and is likely a degenerative process. The formation of these characteristic spheroids within the SNr in close vicinity to GABAergic neurons provides compelling evidence that neurodegeneration is occurring with the SNr in PLAN. Additionally, histologically investigations have shown that the SNr is one the most prominent areas of iron accumulation within patients with NBIA (Schneider, Hardy, & Bhatia, 2012). Interestingly, the formation of these spheroids within the SNr is observed at 60 days of age in homozygous mice; before visible motor symptoms are seen in the mice and before any reduction in dopaminergic function in the SNC. This is suggestive that
neurodegeneration and inclusion formation within the SNr precedes a loss of dopaminergic function in homozygous mice. Another possibility worth noting is that these spheroids are forming in axonal projections of the GABAergic neurons of the GP which project to the SNr. The loss of GABAergic inhibition of the GABAergic neurons of the SNr would lead to an increase in inhibition of the SNc dopaminergic neurons by the GABAergic neurons of the SNr. This theory is appealing because a loss of dopaminergic function which would be seen in this case was seen in the mice. Further research is needed to determine the exact nature of the degeneration however.

The loss of GABAergic activity within the SNr, which serve as tonic inhibitors of the SNc dopaminergic neurons (Alexander et al., 1986; Alexander & Crutcher, 1990; Lee, 2007), is likely to lead to dysregulation of the function of SNc dopaminergic neurons, which could predispose them to degeneration, leading to a loss of dopaminergic function. Further research to confirm this hypothesis is needed however. How the recent findings of Strokin et al. (2012) and Strokin and Reiser (2016 and 2017) of dysfunctional Ca\textsuperscript{2+} handling by astrocytes contributes to the degeneration seen in the SNr still needs to be determined. Does this dysfunction in astrocytes create an environment that contributes to the loss of GABAergic cells, or could similar defects in Ca\textsuperscript{2+} handling occur in neurons and directly contribute to neurodegeneration?

Increased iron levels were seen in the SN of homozygous mice at both 60 and 90 days of age. To our knowledge, iron accumulation has not been previously reported in any models of NBIA (Beck et al., 2011; Brunetti et al., 2012, 2014; Kuo et al., 2005; Shinzawa et al., 2008; Strokin & Reiser, 2016, 2017; Strokin et al., 2012; Wu, Li, Lv, & Zhou, 2009; Zhao et al., 2011) Additionally, the accumulation of iron in SN, but not the midbrain increased slightly with age in the homozygous mice. This detection was enabled through the combination of Cy3 TH staining as well as DAB intensification of the PERLs stain. TH staining allowed for specific selection of the SN, which is notable because iron accumulation in the midbrain was not significantly affected in homozygous mice. Usage of the PERLS stain with methods that could not specifically target the SN would be unable
to detect this difference. Therefore, previous investigations may have lacked the specificity to observe this iron accumulation.

Similar to what was seen in the mice in this study, iron deposition has been shown to increase with disease severity in PD (Adibhatla & Hatcher, 2010). In NBIA, iron accumulation is frequently seen in the BG (Gregory & Hayflick, 2013; Hogarth, 2015). More specific methods such as susceptibility weighted MRI imaging are used early in the disease course in order to be able to see initial iron deposition (Gregory & Hayflick, 2013; Hogarth, 2015). This parallels the findings of our study and suggests methods such as DAB intensification should be used when measuring iron levels to increase the sensitivity of the assay and efforts should be taken to specifically measure the iron levels with the SN in order to be able to more consistently identify the presence of iron accumulation in NBIA models.

3.6. Conclusions

The presence of severe early-onset neurodegenerative symptoms was confirmed in B6.C3 Pla2g6<sup>CM1/J</sup>/Cxrwb mice. This included a pronounced motor deficit in a wire hang test of strength and coordination, but not in tests of general locomotion. Modifications to the wire hang test utilized in previous NBIA studies were suggested, which address some limitations of the test and improve its ability to identify motor deficits in affected mice. Neurodegeneration within the BG was demonstrated in the mice. This presented in the form of a reduction in TH expression within the dopaminergic neurons of the SNc. This loss was shown to be age dependent and significantly correlated with the motor deficit seen in the mice. The widespread formation of spheroids was observed throughout the midbrain of the mice as well as a concentrated degeneration and formation of spheroids based on THY1-YFP was seen with the SNr. The formation of spheroids preceded both the loss of TH in the SNc and the onset of motor symptoms suggesting that early degeneration in PLAN may be within the SNr. A targeted analysis using PERLS/DABs staining revealed a slight increase in iron deposition within the SN. This modified approach was able to detect a slight increase in iron by using Cy3 targeted to TH to directly measure the iron
accumulation of the SN, a method which to our knowledge has not been previously utilized in NBIA.

Collectively, these findings highlight the B6.C3 Pla2g6m2j/CxRwb mice as a useful model of PLAN. These mice present with a severe and early-onset motor deficit, which has been poorly captured by other models of the disease. Furthermore, key hallmarks of NBIA including degeneration and iron accumulation within the BG were demonstrated in these mice. Novel findings of early targeting of the SNr for degeneration in PLAN warrant further investigation. Confirmation of neuronal loss and an exploration as to the causative mechanism of degeneration in the SNr represent two possible areas of future study. To date it is the only available model which captures many of the major hallmarks of NBIA including severe-early onset neurodegeneration, a motor deficit as well as degeneration and iron accumulation within the BG.
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Chapter 4. Systemic Profiling and Long-Term Clinical Investigation of a Case of Phospholipase-A2G6 Associated Neurodegeneration / PARK14 Parkinson’s disease

4.1. Abstract

Phospholipase A2G6-Associated Neurodegeneration (PLAN) is a rapid and devastating, rare, early-onset, monogenic neurodegenerative movement disorder which primarily targets the BG. A selection of cases of PLAN manifest as a dystonia-parkinsonism in early adulthood. Due to common symptomology as well as shared hallmarks of degeneration and iron accumulation within the basal ganglia this presentation of PLAN is also classified as a form of Parkinson’s disease (PD, PARK14). Long-term monitoring of a single patient with PARK14/PLAN; who was being treated by the Vancouver Island Health Authority, through clinical trials with the iron chelator deferiprone (DFP) and the antioxidants alpha-lipoic acid (ALA) and n-acetylcysteine (NAC) was performed in order to improve understanding of the systemic pathophysiology of PLAN/PARK14 and to assess these novel therapies. The patient’s systemic iron levels were largely normal with a 25% reduction in levels of transferrin. A 137% elevations of CRP and 360%, 1500% and 100% elevations of the levels of inflammatory cytokines IL-6, TNFα and IL-10 respectively, highlight a systemic disease burden in PLAN. Systemic elevations in these inflammatory markers as well as an in the oxidative stress marker malondialdehyde were noted over the course of disease progression. Proteomic analysis revealed a series of possible candidate blood-based biomarkers that were abnormally expressed in PLAN including: fibrinogen (90% increased), transthyretin (50% decreased), adiponectin (100% increased), zinc alpha 2-glycoprotein (20% increased) and serum amyloid P (80% increased). Notable increases in both oxidative stress markers (malondialdehyde) and inflammatory markers (IL-6, TNF α and IL-10) occurred between the DFP and antioxidant trials which the patient was untreated. This is suggestive that an increasing disease burden occurs as the disease progresses. Both chelation with DFP and antioxidant therapy with ALA and NAC had no adverse reactions reported. DFP as well as ALA were efficacious in reducing the systemic burden of inflammation; as demonstrated by reduced levels of CRP, IL-6 and TNFα as well
as mitigating increases in malondialdehyde. Neither therapy improved the neurodegenerative symptoms in the patient or slowed disease progression. This may have been partially due to the advanced disease state at the time of the initiation of therapy. However, even in advanced cases these therapies may be efficacious in limiting the systemic disease burden of neurodegeneration in PLAN as was demonstrated in this case study. Despite the lack of efficacy in improving motor symptoms the biomarker investigation in this study highlights a systemic disease burden in PLAN, which was targeted by therapy, as well as the novel candidate biomarkers, fibrinogen, transthyretin, adiponectin, zinc alpha 2-glycoprotein and serum amyloid P.

4.2. Introduction

Phospholipase A2G6-associated neurodegeneration (PLAN) is a member of a group of rare, early-onset, familial, monogenic neurodegenerative disorders known as neurodegeneration with brain iron accumulation (NBIA) (Hogarth 2015). These disorders are grouped due to a shared targeting of the basal ganglia (BG) for degeneration which results in the presentation of extrapyramidal symptoms as well as cognitive and motor dysfunctions. A strongly shared hallmark between these disorders is the accumulation of excess iron within the BG; particularly in the globus pallidus (GP) and substantia Nigra (SN) (Hogarth 2015). Though PLAN is relatively rare with incidences in the range of 1 in 1,000,000, it has strong parallels to more common neurodegenerative disorders; in particular to Parkinson’s disease (PD) (Rouault 2013). Research efforts in PLAN provide insight into two key shared hallmarks of these disorders: neurodegeneration and abnormal iron accumulation inside the BG. Research into better understanding of the underlying mechanisms which lead to dysfunction in NBIA not only benefits the relatively small group of individuals affected by these disorders, but also the larger patient population suffering from disorders featuring neurodegeneration of the BG.

The work of Morgan et al. (2006) determined that PLAN represents a subtype of NBIA, unique from Pantothenate Kinase-Associated Neurodegeneration (PKAN), which is caused by mutations in the PLA2G6 gene encoding the calcium independent
phospholipase A2G6 (iPLA2β). Pathogenic mutations found in PLAN generate enzymatically inactive isoforms of iPLA2β which inhibit the actions of the phospholipase and render it inactive (Morgan et al., 2006). Due to the fact that iPLA2β has been shown to have strong mitochondrial expression (Six & Dennis, 2000) as well as proposed roles in a diverse set of cellular functions including membrane dynamics and homeostasis through phospholipid remodeling, as well as leukotriene and prostaglandin synthesis, apoptosis and inflammation (Morgan et al., 2006; Schneider, Bhatia, & Hardy, 2009; Sun et al., 2010) defects in iPLA2β have the potential to have wide ranging effects in the cellular environment.

Though the most common presentation of PLAN is an extremely early-onset and rapidly progressing form of neurodegeneration known as infantile neuroaxonal dystrophy (INAD) (Gregory & Hayflick, 2013; Hogarth, 2015), PLA2G6 mutations can also present as a progressive dystonia-parkinsonism syndrome which manifests in late adolescence or early adulthood (Hogarth, 2015). The strong similarity between this presentation of PLAN, which includes dystonia-parkinsonism, iron accumulation inside the SN as well as a loss of dopaminergic function, and classical PD has led mutations in PLA2G6 to also be classified as a causative gene in PD; PARK14 (Yoshino et al., 2010; Zhou et al., 2016). The dual characterization of this form of PLAN as both NBIA and PD is also due to the presence of Lewy body and synucleinopathy pathology in idiopathic cases of PLAN/PARK14 PD (Miki et al., 2017; Paisán-Ruiz et al., 2012) as documented in the investigations of Gregory et al. (2008).

4.2.1. Systemic Investigations of PLAN

In contrast to the efforts to observe and investigate the neuropathophysiology of PLAN, research and understanding of the systemic pathophysiology is limited. Preliminary investigations of the systemic state of NBIA using animal models (Kuo et al., 2005), primary cell cultures (Campanella et al., 2012; Santambrogio et al., 2015) as well as metabolic profiling (Leoni et al., 2012), have only been performed in PKAN, another NBIA disorder. Many questions remain unanswered in regard to how degeneration inside the
CNS translates systemically. Additionally, it is still unclear what contributions systemic iron metabolism makes to the iron accumulation and degeneration seen in the BG. Improving understanding of the systemic dysfunction which occurs in these helps to delineate systemic processes that may be occurring and contributing to disease progression. It also provides the opportunity to assess the possibility of a systemic disease burden in patients. Finally, these investigations give the opportunity to explore and evaluate candidate biomarkers which serve as indicators of biological processes, pathogenic process or pharmacological response to therapy. Currently, limitations exist in the available clinical tools in NBIA, such as genetic analyses, MRI and PET scans as well clinical rating scales, due to the expense or training required in their administration and their poor capture of the systemic state. Due to their low cost and ease of access, systemic blood-based biomarkers are well suited to complement these existing methods and provide additional utility in the assessment of NBIA. However, improvements in our systemic understanding of PLAN and other NBIA disorders are necessary to pursue these biomarkers.

4.2.2. Chelation

Though the contribution of iron accumulation with the BG in NBIA is still being elucidated, the potential for accumulating iron to cause chronic tissue damage has been well demonstrated in other iron overload diseases such as thalassemia (Cao and Galanello 2010, Ginzburg and Rivella 2011). Therapeutically chelation has been used to treat iron overload in Thalassemia for more than 20 years (Olivieri et al., 1995). Chelation and removal of excess iron limits the potential for an increased LIP to catalyze the formation of excess ROS, which is highly toxic to the cellular environment; damaging lipids, proteins and DNA. It provides a method for the removal of accumulated iron from within tissues, which is not present endogenously within the body. The efficacy of many chelators has been robustly demonstrated, but research efforts in NBIA and PD have focused on the orally active chelator deferiprone (DFP, 1,2-dimethyl-3-hydroxypyrid-4-one) (Abbruzzese et al., 2011; Devos et al., 2014; Dexter et al., 2010; Fasano et al., 2012; Forni et al., 2008; Ward et al., 1995; Zorzi et al., 2011). Additionally, DFP is membrane permeable (Kakhlon,
Breuer, Munnich, & Cabantchik, 2010; Sohn, Breuer, Munnich, & Cabantchik, 2008) and has been demonstrated to be able to scavenge intracellular iron and redistribute it between nuclei, mitochondria and endosomes as well as delivering it to apotransferrin; transferrin without any iron bound (Sohn et al., 2008). Alongside this iron redistribution between various pools for further use, DFP also is readily excreted in the urine or bile along with its bound iron (Hoffbrand, Cohen, & Hershko, 2003), facilitating the removal of unneeded excess iron. Initial trials in PD rat (Dexter et al., 2010; Ward et al., 1995) and mouse (Devos et al., 2014) models showed the efficacy of DFP in acting across the BBB, which is a key requirement in the treatment of brain iron accumulation. The removal of iron from the SN by these chelators was also able to protect against the loss of DA neurons in these models (Dexter et al., 2010; Ward et al., 1995; Devos et al., 2014).

DFP’s efficacy in targeting brain iron accumulation has also been demonstrated in clinical trials in patients with NBIA (Abbruzzese et al., 2011; Fasano et al., 2012; Forni et al., 2008; Zorzi et al., 2011) and PD (Devos et al., 2014); nearly all of the patients treated showed a substantial reduction in brain iron accumulation. Trials so far in NBIA have been small in scale and somewhat limited in duration NBIA (Abbruzzese et al., 2011; Fasano et al., 2012; Forni et al., 2008; Zorzi et al., 2011), with only a handful of patients receiving therapy and most of them only being treated for a year or less. The trial by Devos et al. (2014) in patients with PD demonstrated the potential for additional efficacy in long term therapy. In all animal model and clinical studies DFP has been demonstrated to be universally well tolerated. However, improvements in motor symptoms were only noted in a selection of patients treated. It appears that the efficacy of DFP in improving motor symptoms may be limited, but further trials are needed to confirm this.

4.2.3. Antioxidant Therapy

Elevated oxidative stress is a commonly noted feature of neurodegeneration. This is highlighted in recent patient studies in PD (Adibhatla & Hatcher, 2010; Mariani, Polidori, Cherubini, & Mecocci, 2005; Núñez et al., 2012) and NBIA (Campanella et al., 2012; Santambrogio et al., 2015). Chronic inflammation is also frequently found in correlation
with the oxidative state (Khansari, Shakiba, & Mahmoudi, 2009), so it is perhaps not surprising that inflammatory processes are also highly implicated in studies of PD (Andican et al., 2012; Chen, O’Reilly, Schwarzschild, & Ascherio, 2007; Nagatsu & Sawada, 2005; Niranjan, 2014). Recently, an emerging avenue of treatment of this oxidative and inflammatory state is the use of various antioxidants. Two strong candidate anti-oxidants are alpha-lipoic acid (ALA) and n-acetylcysteine (NAC). ALA is a potent antioxidant because it promotes the removal of free radicals and increases levels of other natural antioxidants (Packer, Witt, & Tritschler, 1995). Due to the ability of LA to cross the BBB and reduce oxidative stress in the CNS, it has been suggested as a therapy for neurodegenerative diseases (Gilgun-Sherki, Melamed, & Offen, 2001). Studies of NAC administration in Alzheimer, PD and, Gaucher’s disease patients and healthy controls showed that administration of NAC increases brain levels of GSH as assessed by MRI (De Araújo et al., 2011; Holmay et al., 2013; Maczurek et al., 2008) For humans, a number of clinical trials using ALA have been undertaken with no reported adverse effects versus placebo (Cremer, Rabeler, Roberts, & Lynch, 2006; Shay, Moreau, Smith, Smith, & Hagen, 2009). Similarly, NAC has been used in a number of clinical trials including Alzheimer and Parkinson disease (Arakawa & Ito, 2007; Berk, Malhi, Gray, & Dean, 2013). However, information on the use ALA and NAC in NBIA is limited.

4.2.4. Overview

This case study was aimed at the long-term monitoring of a patient with the PARK14 progressive dystonia-parkinsonism presentation of PLAN. Monitoring was paired with investigations into the systemic state of processes typically implicated in NBIA: dysfunctional iron metabolism, chronic oxidative stress and inflammation as well as abnormal lipid metabolism. Interventions with the iron chelator DFP as well as the antioxidants ALA and NAC were also trialed during this period These therapies were targeting abnormal iron accumulation in the BG as well as elevations in oxidative stress and inflammation respectively. This investigation provides novel insight into systemic dysfunction in PLAN and a preliminary characterization of the state of systemic iron
metabolism in this disorder. Additionally, it assesses the efficacy of two emerging therapies in NBIA.

4.3. Methods

4.3.1. Background of PLAN Patient

An overview of early patient background and the initial patient assessment were performed by Agarwal et al. (2012). Briefly, the patient is a 170 cm tall, 28-year-old male of Scandinavian descent weighing 72 kg. The patient’s family history was negative for parkinsonism, but at age 24 he began to show signs of progressive parkinsonism neurodegeneration. Genetic screening of the patient for common PD and NBIA mutations revealed that he had a single pathogenic mutation inside the PLA2G6 gene; an Ala80Thr substitution (Agarwal et al., 2012). Though typically cases involving PLAN are homozygous recessive, INAD and idiopathic NBIA pathology has previously been seen in patients who are compound heterozygous for PLA2G6 mutations (Gregory et al., 2008). This included a patient with a compound heterozygosity where one of the mutations was the Ala80Thr substitution (Gregory et al., 2008). In this patient, the PLA2G6 gene was only sequenced inside of the exons so it remains possible that an intronic mutation leading to a compound heterozygosity is present or that a synergistic heterozygosity is present. Additionally, heterozygous PLA2G6 mutation variants have been seen in cases of PD, though it was not explored whether or not these mutations were causative (Gui et al., 2013). Following identification of the PLA2G6 mutation, MRI scans were performed, which identified signs of abnormally high iron deposition inside the GP and SN consistent with the characteristic “eye of the tiger” presentation (Agarwal et al., 2012). Initial clinical symptomatic presentation involved mild cognitive dysfunction, moderate supranuclear gaze palsy, myoclonus in the hands, pyramidal signs in the lower limbs and lid-opening apraxia. The patient’s Parkinsonism presentation is mild-moderate and largely symmetrical (Agarwal et al., 2012).

At the time of baseline analysis medication used by the patient includes continued dopamine replacement therapy using levidopa/carbidopa at 100.25 mg 5 times a day and
200.5 mg before bedtime. Additionally, treatment of depression using 20 mg a day of Ceprallex (escitaloprom) and 25 mg a day of Trazodone as antidepressant and sleep aid at bedtime.

4.3.2. Study Controls

16 control patients were recruited at the University of Victoria in Victoria, B.C. All controls provided informed consent to participate in this study. The participation of controls was jointly approved by the University of Victoria and the Vancouver Island Health Authority research ethics boards (CREB #J2013-009). Minors under the age of 17 also had informed consent provided by their parents or guardians. For full control demographics see Table 4-1. A single control with a previous history of anorexia nervosa was removed from the control group. for a final control group of 15 patients. See Appendix H for further details on this control.

<table>
<thead>
<tr>
<th>Table 4-1. Demographic characteristics of control group in comparison to a single patient with PLA2G6-Associated Neurodegeneration (PLAN).</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>Control</em> (n = 15)</em>*</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Height (cm)</td>
</tr>
<tr>
<td>Weight (kg)</td>
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<td>Sex</td>
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<tr>
<td>Ethnicity</td>
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<td></td>
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<tr>
<td>BMI</td>
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</tbody>
</table>

*All control values are ± SE

4.3.3. Deferiprone Therapy

The patient underwent a 37-week compassionate use pilot study and research trial of DFP treatment. This trial was jointly approved by the University of Victoria and the Vancouver Island Health Authority research ethics boards (CREB #J2013-009). The patient provided informed consent to this compassionate use research trial. The purpose of this trial was to assess the efficacy of DFP based on improvements to quality of life as well as motor symptoms and its effect on systemic markers of oxidative stress, inflammation and
iron metabolism. An initial dosage of 1000 mg/day (13.8 mg/kg/day) of DFP was used. Patient tolerance was assessed at this dosage and in the absence of notable adverse effects the dose was increased up to 1500 mg/day (20.8 mg/kg/day) and ultimately to a dose of 2000 mg/day (30 mg/kg). Final dosing was administered 4 times daily at 500 mg. DFP was graciously provided for this trial by ApoPharma Inc. For an overview of the study setup see Figure 4-1.

4.3.4. Blood Sample Collection and Processing

All blood samples were collected by a trained phlebotomist. Three major blood draws, at baseline as well as at 19 and 37 weeks of DFP treatment were performed. Blood samples for plasma isolation were collected in heparin treated tubes and serum samples were collected in untreated serum tubes. Plasma sample tubes were spun at 1000 g for 10 min immediately following collection. Following the spin, the plasma layer was removed and aliquoted. Plasma samples were immediately frozen and stored in cryovials at -80°C. Serum tubes were allowed to sit for 40 min for blood clotting. Following clotting tubes were spun at 1000 g and the serum layer was removed and aliquoted. Serum samples were stored in cryovials at -80°C.

4.3.5. Patient Monitoring

Additional blood samples were necessary to monitor for signs of agranulocytosis (as DFP can cause) and collected weekly over the course of the DFP therapy beginning with a baseline assessment immediately prior to the initiation of therapy. This monitoring included the measurement of a complete blood cell count as well as serum ferritin, alanine aminotransferase (a marker of liver function), serum iron levels, TIBC, transferrin saturation, the acute inflammatory protein c-reactive protein (CRP) and lactate dehydrogenase (LDH) as a measure of tissue damage. See Appendix I for a complete list of markers measured through weekly monitoring. All blood samples were collected by a trained phlebotomist. Markers were measured by Island Health Hospital Laboratory Services and LifeLabs® Canada.
4.3.6. Antioxidant Therapy

Monitoring of the patient continued longitudinally following the end of DFP therapy. After a preliminary analysis of the outcome of therapy, antioxidant therapy targeting the increased oxidative stress and inflammation observed in our preliminary investigation was conducted. R-Alpha lipoic acid (ALA) at 1200 mg/day and n-acetylcysteine (NAC) at 1500 mg/day were selected as two trial antioxidants. ALA and NAC were each administered alone in 2-month doses followed by continued combination therapy with both ALA and NAC. ALA and NAC were graciously provided for this trial by Advanced Orthomolecular Research (AOR). For an overview of the antioxidant study setup see Figure 4-1.

4.3.7. Blood Sample Collection and Processing

5 major blood draws were performed during treatment. A preliminary blood draw was performed prior to the initiation of therapy. Additional blood draws were performed following 2 months of therapy with ALA, 2 months of therapy with NAC and 2 months of combination therapy with ALA and NAC. A final blood draw was performed after 1 year of continued combination therapy (ALA and NAC). Blood samples were collected and treated as noted above for the DFP trial.
4.3.8. Biomarker Measurements

In order to limit intraassay variability each sample plate was run with an internal standard curve. Additionally, each plate was run with a mixture of control and patient samples.

Lipid Peroxidation Products

Malondialdehyde

The lipid peroxidation product malondialdehyde (MDA) was quantified using N-Methyl-Phenyl-Indole (NMPI). NMPI reacts and forms a colourimetric product with
malondialdehyde with a peak absorbance at 586 nm. Each plasma sample to be analyzed was thawed on ice and treated with butylated hydroxytoluene (BHT, Sigma) at a final concentration of 5 µM to limit artefactual oxidation. Additionally, all samples remained on ice throughout the assay to further limit oxidation. Thawed samples were spun for 2 min at 500 g and the plasma supernatant was removed in order to minimize the presence of protein precipitate. A solution containing 20% treated plasma sample (v/v), 65% 6.21 mM NMPI (Sigma) in a 3:1 acetonitrile (Caledon): methanol (Caledon) blend and 15% concentrated HCl (Anachemia) was prepared with each sample to be analyzed. The samples were vortexed and incubated at 45 °C for 60 min. Following the incubation, the protein precipitate was removed by centrifugation at 13000 g for 10 min. The supernatant was removed and spun a second time at 13000 g for 10 min to remove any residual precipitate. 200 uL of processed sample was added to each well of a 96-well plate and read at 595 nM using a Perkin Elmer Victor X5 spectrophotometer. Each sample was measured in triplicate.

**Free Malondialdehyde**

Free MDA was measured in plasma samples by removing proteins using a 10 kDA spin filter (Ultra-0.5 mL Centrifugal Filter, Amicon) prior to analysis of MDA. 200 uL of each sample was spun for 55 min at 14000 g using a 10 kDA spin filter. Following this spin, the filtrate was removed and processed for MDA as outlined above. Both of the spins to remove protein precipitate following the acid incubation were omitted for the free MDA samples due to the lack of protein precipitate.

**Standard Curve**

A standard curve was prepared by treating tetramethoxypropane (Sigma) under acidic conditions to liberate MDA. An 11-point standard curve ranging from 0 to 20 µM MDA was prepared and run in duplicate with each plate. All standards were treated as outlined above using standards in place of plasma samples.
**Inflammatory Cytokines**

The cytokines IL-6, IL-10 and TNFα were measured using Invitrogen Ready-Set Go Human Uncoated ELISA Kits. Each sample was slowly thawed from -80 °C on ice and stored on ice throughout its use to limit cytokine degradation. 90 uL of sample was used for each well of a 96-well ELISA plate. Plate coating, secondary antibody treatment and ELISA substrate exposure were all performed according to the manufacturer’s instructions. Plate washes were performed using PBS-Tween 20 (pH 7.5, PBS-Sigma, OmniPur® Tween®20, Sigma). All samples were run in duplicate. A standard curve was run with each plate of samples (Supplementary Table 0-5). Each standard curve size is based on the concentration range in the patient and control samples. The “drc” package in R (Ritz, Baty, Streibig, & Gerhard, 2015) was used to create a 4-parameter logistic regression for each standard curve. The equation of the logistic regression was used to determine the concentration of all of the samples run. See Appendix J for further standard curve details.

**Serum Iron, Total Iron Binding Capacity and Transferrin Saturation**

Each serum sample was split to measure TIBC and serum iron in the same aliquot. One aliquot of each sample was preprocessed for TIBC then an untreated and a processed aliquot from each sample were simultaneously run through the serum iron measurement.

**TIBC Aliquot Preprocessing**

TIBC preprocessing was performed using a modified version of the Total Iron Binding Capacity Assay Kit from Randox Laboratories (cat#-TI1010). This modification was for the analysis of samples in a 96-well plate. Each sample was mixed with excess iron (89.5 µmol/L Fe³⁺) in a 2:1 ratio (iron: sample). Samples were incubated for 30 min at 22 °C to allow for iron binding. Samples were then incubated with an excess of basic magnesium carbonate (~90 mg/sample) for 60 min, with frequent mixing, to bind any remaining unbound iron. Following incubation, all samples were spun at 1500 g for 10 min. The supernatant was removed and spun for 5 min at 1500 g to ensure no residual magnesium carbonate remained. Following the second spin the supernatant was processed as outlined below for the serum iron samples.
Serum Iron Measurement

Serum iron was measured using a modified version of the Serum Iron Assay from Randox Laboratories (cat#-TISI257). This modification was for the analysis of samples in a 96-well plate. Samples were treated with ascorbic acid (1.3 mol/L) and an acetate buffer in DMSO (0.087 mol/L, pH 4.65) in order to dissociate iron-bound to proteins and reduce it to a ferrous state. A final mixture of 3.8% ascorbic acid, 19.8% sample and 77% buffer was used for each sample in a 96-well plate. See Appendix K for adjusted measurement volumes for use in a 96-well plate. The absorbance of each well was read at 595 nm. Following this initial reading an equal volume to the volume of ascorbic acid added (3.8%) of chromogen (Ferene) was added to each well. The plate was mixed briefly on a shaker and then incubated at 22°C for 15 min. Following this incubation, a secondary reading was taken at 595 nm. The background absorbance was subtracted from the secondary reading in order to normalize for potential differences in serum background such as the presence of excess protein or protein particulate.

Standard Curve

A 7-point standard curve ranging from 36.38 µmol/L to 0 µmol/L Fe³⁺ was prepared using a serial dilution of an included iron standard and run with each plate of samples. Both the serum iron and TIBC aliquots were run in duplicate on the same plate. Percent transferrin saturation was calculated by dividing the serum iron by the TIBC.

University of Victoria Genome BC Proteomic Center Proteomic Biomarker Panel

A final panel of 37 plasma proteins was measured using multiple reaction monitoring (MRM) mass spectrometry proteomics at the University of Victoria Genome B.C. proteomic center in plasma samples. See Appendix L for a full list and overview of the proteins measured. MRM was chosen as a rapid and highly specific method to measure multiple blood plasma proteins for analysis as biomarkers from patients. Quantification of peptides representing target proteins was ensured through the inclusion of stable isotope-labeled standard (SIS) peptides in the analysis. All peptides (light and SIS) were synthesized via Fmoc chemistry, purified (through RP-HPLC with subsequent assessment
by MALDI-TOF-MS, and characterized via amino acid analysis, and capillary zone electrophoresis. For additional details on the setup and utilization of this MRM method see Appendix M.

**Sample Preparation**

Chemicals and reagents were obtained from commercial sources at the highest purities available. All solvents (including water, acetonitrile, and methanol) were LC–MS grade and acquired from Sigma Aldrich.

All plasma samples were prepared using a modified version of the protocol previously outlined by Percy, Chambers, Yang, Hardie, & Borchers (2014). All liquid handling steps were automated and performed with a Tecan Freedom EVO 150. Denaturation and reduction of plasma proteins was performed simultaneously at 37 °C for 30 min using a mixture of 9 M urea, 300 mM Tris and 20 mM dithiothreitol in Tris buffer (pH 8.0). Following denaturation 100 mM iodoacetamide was added for 30 min at room temperature in the dark to alkylate sulfhydryl groups. Proteolytic digestion of processed proteins was performed using a 20:1 ratio of sample protein to Worthington TPCK-treated trypsin from bovine pancreas. Digestion proceeded overnight (~18 hours) at 37 °C. Enzymatic digestion was halted using the addition of a 1% formic acid solution as well as an acidified SIS peptide mixture; which was composed of the synthetic tryptic peptides utilized as surrogates for the target proteins. The SIS peptide mixture was prepared to be “concentration balanced” so that the SIS peptide concentration added was nearly equal to estimated NAT peptide concentration. This yields nearly equal SIS/natural plasma protein peak areas when spiked into the plasma digest. The concentration estimates for the SIS peptides were based on previous validation which obtained peak area measurements from the analysis of equimolar SIS-peptide mixture in plasma samples. Following the preparation all of the samples as well as the standard curve and QC curve were concentrated and desalted via solid phase extraction using Waters Oasis u-HLB plates. They were eluted with 0.1% formic acid and 55% acetonitrile.
Following elution, the samples were lyophilized to dryness and resolubilized in 0.1% formic acid (final concentration of 0.5 ug/uL) and stored at -80 °C until analysis.

**Standard Curve Preparation**

A specialized standard curve composed of synthetic unlabelled (light) tryptic peptides corresponding to each target protein was prepared and run with the samples. For this curve the light peptides are serially diluted to give an 8-point standard curve covering a 1000-fold concentration range starting at the lower limit of quantification (LLOQ) of each peptide. Additionally, a separate 3-point dilution series (at 4X, 50X, and 500X the LLOQ for each peptide) was prepared as a quality control (QC) check for the standard curve. Both the standard curve and QC curve were prepared in demethylated (to shift their masses) human plasma digest. The standard curve was injected in singlicate from the lowest to the highest point on the curve. The QC curve was injected in triplicate. The curve for each peptide was considered valid if it was within 80-120% accuracy range of the QC standards.

**LC-MRM/MS Equipment and Conditions**

Twenty microliter injections of the plasma tryptic digests were separated with a Zorbax Eclipse Plus RP-UHPLC column (2.1 x 150 mm, 1.8 µm particle diameter; Agilent) that was contained within a 1290 Infinity system (Agilent). See Appendix M for LC system parameters. The LC system was interfaced to a triple quadrupole mass spectrometer (Agilent 6490) via a standard-flow ESI source, operated in the positive ion mode. See Appendix M for MRM acquisition parameters. Specific LC-MS acquisition parameters were employed for optimal peptide ionization/fragmentation and scheduled MRM. The peptide optimizations were empirically optimized previously by direct infusion of the purified SIS peptides.

**Quantitative Analysis**

The MRM data was visualized and examined with Skyline Quantitative Analysis software (version 3.6.1.10556, University of Washington). The standard curve for each peptide was used to calculate the peptide concentration in fmol/ul of plasma in the
patient samples through linear regression. For additional data analysis details for this MRM procedure see Appendix D.


dsSISCAPA Proteomic Analysis

Additional proteomic analysis of hemoglobin alpha chain, c-reactive protein (CRP) and soluble transferrin receptor (sTfR) was performed in plasma samples by SISCAPA Assay Technologies in Victoria, B.C. using immunoassay isolation of target peptides followed by MRM proteomics as previously described by Razavi et al. (2016). Samples were digested using an addition only digestion protocol. Ten microliters of plasma sample was added to each well of a 96-well plate. Each well had a pre-dried mixture of urea (Sigma), TCEP (pH 7.0, ThermoFisher) and Trizma1 pre-set crystals (Sigma). These were set to have a final concentration when solubilized of 9 M, 0.05 M and 0.2 M respectively. All further liquid handling steps were performed using an Agilent Bravo Automated Liquid Handling Platform. Initial digestion occurred during a 30-minute incubation at room temperature with vigorous shaking (1000 rpm). Ten microliters of 0.1M IAM was added and samples were incubated for 10 min at room temperature in the dark. Trypsin digestion was achieved by diluting samples 1:9 with 0.2 M Trizma buffer (pH 8.1) and adding 10 mL of 7.3 mg/mL trypsin (Worthington) in 10 mM HCl for an enzyme: protein ratio of 1:10. Samples were incubated for 2 hours at 37 °C. Tryptic cleavage was halted using 10 uL of 0.2 mg/mL tosyl-L-lysine chloromethyl ketone (sigma) in 10 mM HCl. Targeted proteins were capture using protein g-coated magnetic beads prepared as previously described (Razavi et al., 2016). Samples were spiked with heavy isotope labeled peptides corresponding to the targeted proteins. Captured plasma proteins, heavy peptides and beads were collected using rare earth magnets. The supernatants were discard and beads were resuspended in PBS/0.03% CHAPS. Beads were shaken vigorously in the sample plate for 1 hour at 1000 rpm. The beads were subsequently washed three times in PBS/0.03% CHAPS. Proteins were eluted using 0.5% FA/0.03% CHAPS into a 96-well plate. Plates were then run on an LC-MS/MS platform consisting of a 1290 Infinity UHPLC coupled to a 6490 TQMS using a JetStream interface (Agilent Technologies, CA). For LC-MS/MS parameters and running conditions see Razavi et al. (2016). Data
acquisition and analysis was performed using MassHunter Software (Agilent) as outlined by Razavi et al. (2016).

4.3.9. **Data Analyses**

All statistical analyses were conducted using R (R Core Team, 2016). Data was initially plotted and normality was tested based on outliers, ranges and distributions. Summary statistics including mean, SD, SE and 95% confidence intervals were then calculated for the data for control patients. The PLAN patient was explored visually at baseline in comparison to the mean and distribution of the control patient group. Additional visual comparisons of the PLAN patient over the course of DFP and antioxidant therapy were also performed. Results were presented graphically using the R packages “ggplot2” (Wickham, 2009) and “gridExtra” (Auguie, 2016).

4.4. **Results**

4.4.1. **Baseline Investigations and Deferiprone Therapy**

*Monitoring*

No significant adverse reactions were reported during 37 weeks of DFP treatment. Plasma levels of the liver health marker ALT and plasma expression of LDH remained within the normal range (Data not shown). Additionally, no signs of neutropenia or agranulocytosis were noted in the patient. Slight difficulties with the administration of the four times daily treatment schedule of oral deferiprone occurred due to minor dysphagia in the patient.

A pre-DFP-trial UPDRS score ranked the severity of the patient’s symptoms as mild to moderate (Agarwal et al., 2012) with a Global Deterioration Score (GDS) of 4 to 5. Over the next three years during treatment with DFP as well as ALA and NAC, there was a slow but steady deterioration in his motor abilities, with increased dystonia. The patient’s condition deteriorated to the point that he required placement in a long-term care facility. By the time of the institution of the antioxidant trial he was bedridden. By the end of the antioxidant trial he was mute; with a maximum Global Deterioration Score (GDS) of seven.
Iron Metabolism

No notable difference was seen in the serum iron levels in the PLAN patient. Serum iron, TIBC and transferrin saturation were all within the reference range and were largely unaffected by 37 weeks of DFP treatment (Table 4-2). Serum ferritin was reduced by 37 weeks of deferiprone treatment, but levels remained within the reference range (Table 4-2). Proteomic analyses showed a reduction in the plasma levels of transferrin in the patient which matched to the extreme low end of the control range (Figure 4-2A), elevation in the levels of alpha hemoglobin above the control range (Table 4-2) and an elevation in sTfR beyond the 95% CI of the control mean (Figure 4-2B). Elevations in sTfR were notably reduced over 37 weeks of deferiprone therapy.

Table 4-2. Iron profile of a patient with PLA2G6-Associated Neurodegeneration (PLAN) at baseline and following 37 weeks of deferiprone therapy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control* (n = 15)</th>
<th>PLAN Baseline (n = 1)</th>
<th>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Ceruloplasmin (fmol/uL)</td>
<td>1104 ± 64.62</td>
<td>1130.1</td>
<td>929.38</td>
</tr>
<tr>
<td>Plasma Haptoglobin (fmol/uL)</td>
<td>15540 ± 1905.56</td>
<td>36892</td>
<td>21743</td>
</tr>
<tr>
<td>Plasma Hemopexin (fmol/uL)</td>
<td>8280 ± 285.35</td>
<td>8782.2</td>
<td>7335.3</td>
</tr>
<tr>
<td>Plasma Transferrin (fmol/uL)</td>
<td>17150 ± 762.91</td>
<td>13286</td>
<td>13135</td>
</tr>
<tr>
<td>Plasma Hemoglobin Alpha Chain</td>
<td>7.29 ± 0.69 x10^-3</td>
<td>10.9 x10^-3</td>
<td>11.4 x10^-3</td>
</tr>
<tr>
<td>(Normalized Peak Area Ratio)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Soluble Transferrin Receptor</td>
<td>2.84 ± 0.35</td>
<td>3.77</td>
<td>2.94</td>
</tr>
<tr>
<td>(mg/L)</td>
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<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Reference Range</th>
<th>PLAN Baseline (n = 1)</th>
<th>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</th>
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</thead>
<tbody>
<tr>
<td>Plasma Hemoglobin (g/L)</td>
<td>133-165</td>
<td>163.0</td>
<td>151</td>
</tr>
<tr>
<td>Serum Ferritin (µg/L)</td>
<td>15-300</td>
<td>86.0</td>
<td>26</td>
</tr>
<tr>
<td>Serum Iron (µmol/L)</td>
<td>10-33</td>
<td>23.0</td>
<td>23.0</td>
</tr>
<tr>
<td>Total Iron Binding Capacity (µmol/L)</td>
<td>37-72</td>
<td>54.0</td>
<td>60.0</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Transferrin Saturation (%)</td>
<td>20-55</td>
<td>0.43</td>
<td>0.380</td>
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</table>

*All control values are ± SE

**Figure 4-2.** Plasma levels of the iron trafficking protein transferrin (A) and soluble transferrin receptor (B) in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and over the course of 37 weeks of deferiprone (DFP) therapy relative to controls (n = 15); as measured by multiple reaction monitoring mass spectrometry proteomics at the University of Victoria Genome B.C. proteomic center and SISCAPA respectively. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma transferrin levels were low in the patient with PLAN relative to the control population and weren’t notably affected by therapy. Plasma soluble transferrin receptor levels were elevated in the patient relative to the control population and were reduced over the course of therapy.

**Systemic Disease Burden**

A strong systemic inflammatory disease burden was seen at baseline in the patient (Table 4-3). The levels of the acute inflammatory protein CRP as measured by proteomics were outside the 95% confidence interval of the control mean (Figure 4-3A). Elevated CRP levels of 4.0 mg/L were also seen in an independent laboratory diagnostic test (See Supplementary Table 0-4 for reference range). Similarly, elevations of the levels of the inflammatory cytokines IL-6 (Figure 4-3B), IL-10 (Figure 4-3C) and TNFα (Figure 4-3D) beyond the control range were also observed. All four of these inflammatory markers were reduced by 37 weeks of DFP therapy.
**Table 4-3.** Systemic oxidative and inflammatory disease burden of a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and following 37 weeks of deferiprone therapy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control* (n = 15)</th>
<th>PLAN Baseline (n = 1)</th>
<th>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein (Normalized Peak Area Ratio)</td>
<td>0.098 ± 0.054</td>
<td>0.23</td>
<td>0.072</td>
</tr>
<tr>
<td>Malondialdehyde (µM)</td>
<td>2.27 ± 0.27</td>
<td>3.31</td>
<td>5.54</td>
</tr>
<tr>
<td>Free Malondialdehyde (µM)</td>
<td>0.45 ± 0.054</td>
<td>0.054</td>
<td>0.35</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>4.73</td>
<td>3.02</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.84 ± 0.22</td>
<td>6.75</td>
<td>3.59</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.33 ± 0.36</td>
<td>39.30</td>
<td>16.10</td>
</tr>
</tbody>
</table>

*All control values are ± SE

**Figure 4-3.** Plasma expression of the acute inflammatory protein CRP (A), and the inflammatory cytokines IL-6 (B), IL-10 (C) and TNFα (D) in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and over the course of 37 weeks of deferiprone (DFP) therapy relative to controls (n = 15); as measured by SISCAPA immunoassay enhanced multiple reaction monitoring mass spectrometry proteomics (A) and ELISA (B-D). Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. All four inflammatory markers were notably elevated in the patient with PLAN and were reduced over the course of 37 weeks of treatment.
Elevated levels of glutathione peroxidase were seen matching the extreme upper limit of the control range (Figure 4-4A). The levels of glutathione peroxidase fell steadily over the course of 37 weeks of deferiprone therapy. A minor increase in the plasma levels of MDA were seen at baseline (Figure 4-4B). These levels were initially reduced by DFP therapy, but it had risen by the end of 37 weeks of treatment. The levels of free MDA were also markedly reduced early in DFP therapy (Figure 4-4C). These levels had risen back to the control mean by the end of 37 weeks of deferiprone therapy.

Figure 4-4. Plasma expression of the antioxidant enzyme glutathione peroxidase (A), and the lipid peroxidation products malondialdehyde (MDA, B) and free MDA (C) in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and over the course of 37 weeks of deferiprone (DFP) therapy relative to controls (n = 15); as measured by UVic Genome BC proteomic center multiple reaction monitoring mass spectrometry proteomics (A) and using a NMPI based colourimetric assay (B and C). Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma glutathione peroxidase levels were elevated in the patient with PLAN relative to the control population and declined over the course of 37 weeks of deferiprone therapy. Plasma MDA levels were slightly elevated above the median of the control population in the patient with PLAN. Treatment with deferiprone initially reduced MDA levels, but the levels rose notably by the end of 37 weeks of treatment. Plasma free MDA levels were not noticeably different from the control median. Treatment with deferiprone initially reduced free MDA levels, but the levels rose notably by the end of treatment.
**Lipid Metabolism**

Signs of altered lipid metabolism were seen in the patient at baseline (Supplementary Table 0-8). Levels of adiponectin and zinc alpha 2 glycoprotein, which regulate the utilization of fatty acid stores in adipocytes were raised beyond the control patient range (Supplementary Table 0-8). 37 weeks of deferiprone therapy lowered the expression levels of these proteins towards the control range. Additionally, the levels of numerous apolipoproteins including apolipoprotein Al, Cl, CII and M were reduced below control levels (Supplementary Table 0-8). Deferiprone therapy raised the expression levels of Apo AI, Cl and CII towards the control range (Supplementary Table 0-8).

**Other Markers of Interest**

A small group of other potential proteins of interest were noted, which showed levels well outside the control range (Supplementary Table 0-8). These included elevations all three chains of fibrinogen, alpha-1 antitrypsin and serum amyloid-P (Supplementary Table 0-8). 37 weeks of DFP therapy reduced the levels of these proteins to the control range. Additionally, transthyretin levels were reduced below the control range (Supplementary Table 0-8). 37 weeks of DFP therapy raised the plasma levels of transthyretin into the control range. See Appendix N for full baseline proteomic panel results as well as the results at the end of DFP therapy for the PLAN patient.

**Impact of Deferiprone Therapy**

As previously outlined, no improvement in the patient’s motor symptoms was noted during 37 weeks of DFP therapy. Due to the lack of notable improvement in the patient’s motor symptoms following therapy it was elected to not perform an end of trial UPDRS. See Appendix N for full DFP trial biomarker results.

**4.4.2. Antioxidant Therapy**

**Pre-Trial Assessment and Monitoring**

The severity of the patient’s motor symptoms had progressed between the end of deferiprone therapy and the initiation of the antioxidant trial. The patient was no longer able to walk unaided and required the use of the wheelchair. Additionally, the patient had
been placed into a long-term care facility. Mild cognitive decline, dysarthria and dysphagia were also observed in the patient. No adverse reactions were reported in the patient during therapy. Some difficulty was encountered with oral dosing of ALA and NAC due to dysphagia in the patient.

*Iron Metabolism*

No significant changes in the iron panel measured at baseline (Table 4-2) were seen in the patient at the start of anti-oxidant therapy (Supplementary Table 0-9). Additionally, no changes were observed during the course of anti-oxidant therapy (Supplementary Table 0-9).

*Systemic Disease Burden*

The plasma levels of the inflammatory cytokines IL-6, IL-10 and TNFα rose back to pre-DFP treatment levels during the interval between the end of DFP therapy and the start of antioxidant therapy (Table 4-4). Minor reductions in IL-6 (Figure 4-5A), IL-10 (Figure 4-5B) and TNFα (Figure 4-5C) were seen during 2 months of treatment with ALA. No change was seen during 2 months of treatment with NAC. 16 months of therapy with a combination of ALA and NAC showed a strong reduction in all 3 cytokines towards the control range. A possible acute inflammatory event occurred as measured by elevations in CRP at the end of 16 months of therapy (Table 4-4).

Plasma glutathione peroxidase levels were reduced from baseline levels (Table 4-4) and MDA levels were elevated from baseline levels (Table 4-4) in the patient at the start of antioxidant therapy. Treatment with 2 months of ALA raised plasma glutathione peroxidase expression back to baseline levels (Figure 4-6A). Continued treatment with both 2 months of NAC and 16 months of NAC and ALA maintained this increase in glutathione peroxidase. Treatment with 2 months of ALA prevented further increased in plasma MDA in the patient (Figure 4-6B). These levels rose drastically during 2 months of therapy with NAC. Combination therapy with NAC and ALA mitigated further increases in MDA. No significant trend in free MDA was seen in the patient (Figure 4-6C) aside from a
single possible acute oxidative stress event measured at the end of 2 months of therapy with ALA and NAC.

**Table 4-4.** Systemic oxidative and inflammatory disease burden of a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) prior to the initiation of antioxidant therapy and at the end of 20 months of therapy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control* (n = 15)</th>
<th>PLAN Pre-Antioxidant Trial (n = 1)</th>
<th>PLAN End of Antioxidant Trial (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein</td>
<td>0.098 ± 0.054</td>
<td>0.026</td>
<td>0.94</td>
</tr>
<tr>
<td>(Normalized Peak Area Ratio)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Malondialdehyde (µM)</td>
<td>2.27 ± 0.27</td>
<td>4.06</td>
<td>6.87</td>
</tr>
<tr>
<td>Free Malondialdehyde (µM)</td>
<td>0.45 ± 0.054</td>
<td>0.68</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>4.98</td>
<td>3.27</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.84 ± 0.22</td>
<td>6.37</td>
<td>3.24</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.33 ± 0.36</td>
<td>47.16</td>
<td>14.27</td>
</tr>
</tbody>
</table>

*All control values are ± SE

**Lipid Metabolism**

No notable changes were seen in lipid metabolism associated proteins prior to the initiation of antioxidant therapy or during 20 months of antioxidant therapy (Supplementary Table 0-9). See Appendix N for further details of the expression levels of lipid associated proteins over the course of antioxidant therapy.
Figure 4-5. Plasma expression of the inflammatory cytokines IL-6 (A), IL-10 (B) and TNFα (C) in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and over the course of 20 months of antioxidant therapy (See Figure 4-1 for details of antioxidant trial setup) relative to controls (n = 15); as measured by ELISA. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. All three cytokines were elevated prior to the initiation of therapy. Initial 2 months of treatment with alpha-lipoic acid (ALA) reduced all 3 inflammatory cytokines. 2 months of treatment with n-acetylcysteine (NAC) had no notably affect on the inflammatory cytokines. Continued therapy with a combination of NAC and ALA for 16 months caused a pronounced decrease in these inflammatory cytokines to levels approaching the control range.
Figure 4-6. Plasma expression of the antioxidant enzyme glutathione peroxidase (A), and the lipid peroxidation products malondialdehyde (MDA, B) and free MDA (C) in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN) at baseline and over the course of 20 months of antioxidant therapy (See Figure 1) relative to controls (n = 15); as measured by UVic Genome BC proteomic center multiple reaction monitoring mass spectrometry proteomics (A) and using a NMPI based colourimetric assay (B and C). Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma glutathione peroxidase levels were elevated in the patient with PLAN relative to the control population at baseline. These levels had been reduced prior to the initiation of antioxidant therapy. Antioxidant therapy increased and maintained an increase of glutathione peroxidase levels. Plasma MDA levels were slightly elevated above the median of the control population in the patient with PLAN at baseline. These levels had risen steadily since the initiation of monitoring and were notably elevated prior to the initiation of antioxidant therapy. Treatment with alpha-lipoic acid (ALA) for 2 months prevented further increases in MDA levels, an effect that was not seen following n-acetylcysteine (NAC) treatment. Long-term combination therapy with ALA and NAC was also effective in limiting increases in MDA. No notable trend was seen in the levels of free MDA aside from a single, possible acute oxidative stress event.
Other Markers of Interest

No notable changes were seen in the other potential markers of interest (Supplementary Table 0-9) prior to the initiation of antioxidant therapy or during 20 months of antioxidant therapy (Supplementary Table 0-9). See Appendix E for further details of the expression levels of other proteins of interest over the course of antioxidant therapy.

Impact of Antioxidant Therapy

A steady neurodegenerative decline was seen in the patient over the course of antioxidant therapy. Motor symptoms, cognitive decline, dysarthria and dysphagia all became more pronounced. See Appendix N for a highlight of antioxidant trial biomarker results.

4.5. Discussion

4.5.1. Baseline Assessment

This case study provides unique insight into the systemic disease state and disease progression from the long-term monitoring of a patient with the PARK14 progressive dystonia-parkinsonism presentation of PLAN. Despite previous signs of brain iron accumulation in this patient (Agarwal et al., 2012) he had normal systemic iron levels based on hemoglobin concentration, serum iron, TIBC and transferrin saturation. Alterations were seen in systemic iron trafficking proteins however including decreased Tf and elevations in sTfR. The relationship between alterations in these systemic iron trafficking proteins and iron accumulation in the BG still needs to be determined, but it is suggestive of a possible mechanism of iron trafficking into the brain. There are minimal signs of an increased iron demand systemically, so the possibility exists that Tfr expression is increased at the BBB resulting in an increase in sTfR in the plasma. This would be pair with a slight rise in the sTfR which is being released from the BBB. This would correspond with a decrease in Tf levels as it is turned over at the BBB during the delivery of iron. Interestingly, a reduction in systemic transferrin levels is also a common finding in PD and was noted in the work of Logroscino et al. (1997) and Tórsdóttir,
Kristinsson, Sveinbjörnsdóttir, Snaedal, & Jóhannesson (1999). Whether these reductions are due to changes of iron trafficking into the CNS still need to be determined.

A systemic burden of neurodegeneration was also seen in the patient. Large elevations of the proinflammatory cytokines IL-6 and TNFα were seen. CRP levels were also elevated by two separate measures. Interestingly, this was accompanied by elevations in the anti-inflammatory cytokine IL-10, possibly as an immune response to limit this chronic inflammation (Mizoguchi, Mizoguchi, Takedatsu, Blumberg, & Bhan, 2002). Only minor signs of oxidative stress were seen, primarily in the form of a slight elevation in MDA. Elevations in both oxidative stress and inflammation have been previously reported systemically in PD (Andican et al., 2012; Chen et al., 2009; Chen et al., 2007), which is further suggestive of a systemic burden in these disorders.

Both elevations in adiponectin and zinc alpha-2 glycoprotein (ZAG) and reductions in apolipoproteins highlight possible alterations in systemic lipid metabolism in these patients. Recent work by Leoni et al. (2012) revealed global deficits in lipid metabolic pathways associated with CoA in PKAN. Similar defects could also occur in PLAN due to a deficit in iPLA2β. The link between iPLA2β and these pathways is not clear however. The activity and regulation of these proteins is connected to lipid metabolism and the regulation of body weight as well as systemic inflammatory processes. Adiponectin upregulates adipocyte formation and favours lipid deposition (Turer & Scherer, 2012), where as ZAG has proposed roles in the downregulation of lipogenic enzymes and the upregulation of lipolytic enzymes (Gong et al., 2009). Elevations in these two proteins could result in changes in the overall levels of HDL and LDL and their associated carrier lipoproteins. Expression levels of both adiponectin and ZAG have also been reported to be reduced in the presence of ongoing inflammatory signals such as IL-6 and TNFα (Bao et al., 2005; Esteve, Ricart, & Fernández-Real, 2009). Adiponectin has an additional role in the regulation of inflammation. It has been demonstrated to be able to suppress inflammatory responses in a variety of cell types including macrophages (Turer & Scherer, 2012). Interestingly, neither of these phenomena were seen in the PLAN patient. Adiponectin was unable to suppress chronic inflammation. Also, elevations of TNFα and
IL-6 in the patient did not reduce the increased systemic levels of adiponectin or ZAG. This is suggestive that dysfunction in our current understanding of their regulation and activity occurs in PLAN. Ultimately, the regulation of these proteins in a disease state such as NBIA still needs to be fully explored. The simultaneous occurrence of factors affecting these systems in PLAN including chronic inflammation, dysfunction in proteins involved in lipid metabolism and weight loss and muscle wasting raise the possibility of a unique expression profile in patients which may be indicative of PLAN.

Other notable disruptions that were seen in the PLAN patient include elevations in fibrinogen and serum amyloid P and reductions in transthyretin (TTH). Interestingly all three of these proteins were highlighted by a recent large scale systemic review of biomarker studies in PD as potential systemic biomarkers (Alberio et al., 2013). It was proposed that both chronic inflammation as well as alterations in the permeability of the BBB allowing leakage may be responsible for elevations of serum amyloid P in PD (H.-M. Chen, Lin, & Wang, 2011). Recent investigations of TTH have led to proposed roles for it in a wide range of disorders ranging from amyloidosis to Alzheimer’s disease and PD. Its involvement in these disorders has been attributed to a wide range of cellular and metabolic activities including retinol and thyroid hormone metabolism as well as its potential to form protein aggregates (Fleming, Nunes, & Sousa, 2009). Interestingly it is thought to be protective against amyloid beta plaque formation in Alzheimer’s disease (Velayudhan et al., 2012). Low levels of TTH, which are also seen in the patient with PLAN have been prosed as a biomarker in Alzheimer’s disease that correlates inversely with disease severity (Han et al., 2011; Velayudhan et al., 2012). Elevations of fibrinogen have been previously reported to be associated with an increased risk of PD (Wong et al., 2010). It is also reported to pass through the BBB and caused damage via fibrin deposition in Alzheimer’s disease (Ryu & McLarnon, 2009). It appears that alterations in these proteins in NBIA may be related to shared disease processes with disorders such as Alzheimer’s disease and PD. Further investigation with a larger patient group is needed to determine the prevalence of alterations in these markers in PLAN and other NBIA disorders in order to assess their potential as systemic markers of NBIA.
4.5.2. Efficacy of Deferiprone Therapy

During the DFP trial physician clinical assessment noted a steady decline in the patient, based on the severity of motor and cognitive symptoms. Progression in disease severity was also noted. Additionally, no adverse effects were reported. Due to the lack of change in presentation a full UPDRS was not performed at the end of the study. Similarly, the patient was unable to comfortably undergo a post trial MRI. Based on previous trials, 37 weeks of therapy may have been sufficient to cause some iron reduction in the BG. In trials by Forni et al. (2008) and Abbruzzese et al. (2011) a significant reduction in brain iron content visible on MRI was seen after 6 months of treatment. Notably the work of Devos et al. (2014) showed that additional treatment beyond 6 months had a further efficacy in reducing iron content. Though it is unclear if further treatment would have resulted in an improvement in motor symptoms in the patient. Another key finding of the work by Devos et al. (2014) was the fact that early intervention with DFP appeared to greatly improve its efficacy in providing improvement in motor symptoms. During early intervention sufficient functionality may remain within the BG to allow for some recovery of motor function. It is likely that as neurodegeneration of the BG in PLAN progresses fewer functional neurons would remain to enable this recovery. Which could explain some of the reduced efficacy in more severely affected patients.

Notably, a small difficulty occurred in the dosing of DFP in the patient. Ideally DFP is administered multiple times daily to maintain concentrations throughout the day. However due to difficulties swallowing due to minor dysphagia this strategy of administration proved cumbersome in the patient. Alternate dosing strategies may need to be explored in patients with dysphagia.

No significant impact was seen during DFP therapy on serum iron, TIBC or transferrin saturation. A drop in serum ferritin was seen in the patient, but the value remained within the normal range. A similar drop in serum ferritin was observed by Devos et al. (2014) during deferiprone treatment of a group of patients with NBIA. A reduction in serum ferritin could have been due to a reduction or redistribution of hepatic iron stores, as was proposed by Devos et al., 2014). Transferrin levels remained low following
therapy, but sTfR levels dropped back down to the control range. Overall, the therapy was well tolerated with minimal impact on systemic iron levels. A notable reduction was seen in all of the inflammatory markers measured in this study. Reductions in the proinflammatory markers IL-6, TNFα and CRP were accompanied by reduced levels of IL-10, which likely dropped in response to decreased inflammation. DFP was able to strongly mitigate chronic inflammation, though levels still remained raised above the control population. In this trial DFP was less efficacious in mitigating oxidative stress. Some impact was seen early in the trial, reducing both free and total MDA levels. However, these levels subsequently spiked with prolonged therapy. This is rise and the continued elevation of the inflammatory cytokines may be related to the continued neurodegenerative disease progression in the patient. DFP therapy also reduced abnormalities seen in a number of proteomic markers assessed in this study. This included reductions in fibrinogen, ZAG and adiponectin as well as elevations in TTH. The impact of these changes on the disease state in PLAN still need to be determined.

4.5.3. Efficacy of Antioxidant Therapy

Antioxidant therapy was focused on the mitigation of this ongoing inflammatory and oxidative burden in the patient. Levels of IL-6, TNFα and MDA all rose considerably following the conclusion of DFP therapy. This increase corresponded with increasing severity of motor symptoms in the patient. Highlighting an increased oxidative and inflammatory burden during the progression of PLAN.

In this pilot trial of antioxidant therapy in NBIA, treatment with ALA, NAC and combination therapy with ALA + NAC were well tolerated by the patient with no adverse side effects reported. Initial therapy with ALA as well as long-term combination therapy with ALA and NAC were efficacious in lowering chronically elevated level of IL-6 and TNFα. It also mitigated further elevations of MDA during disease progression. Therapy with NAC alone did not appear to be effective in targeting oxidative stress or inflammation in the patient. No impact was seen in the presentation of neurodegenerative symptoms in the patient during antioxidant therapy. Disease progression continued during the course of
this trial, with both motor and non-motor symptoms becoming more pronounced. The patient remained in assisted care and was largely unable to perform basic activities, such as sitting up and eating, unaided. Similar trials in PD have revealed limitations to the efficacy of antioxidant therapies (Jin et al., 2014). Despite promising preclinical evidence (Jin et al., 2014) therapeutic intervention using antioxidants has had no impact (Snow et al., 2010) or limited impact (Hauser, Lyons, McClain, Carter, & Perlmutter, 2009) in previous trials in patients with PD. A study into the administration of high doses of tocopherol and ascorbate to patients with PD did show efficacy in slowing disease progression, but not improving motor symptoms (Fahn, 1991). Notably, this study provided evidence that early administration of antioxidants may improve their therapeutic benefit (Fahn, 1991). This closely parallels the findings of Devos et al. (2014) in the administration of DFP chelation therapy; early intervention may provide more benefit, due to the intervention occurring when damage to the BG is less pronounced (i.e. more function remains to be protected). This phenomenon may help to explain some of the lack of efficacy observed in the trial in the improvement of motor symptoms in the patient; due to his advanced disease state, but further investigations are needed to determine the importance of the timing of therapeutic intervention.

4.6. Conclusions

This investigation in a patient with the dystonia-parkinsonism presentation of PLAN revealed that brain iron accumulation NBIA has minimal impact on systemic serum iron levels, TIBC and transferrin saturation. Preliminary evidence of alterations in the trafficking protein pair Tf and sTfR receptor were seen. A systemic burden of chronic inflammation and minor increases in oxidative stress was seen in the patient. Proteomic investigation revealed possible alterations in systemic lipid metabolism including alterations in the expression of adiponectin and ZAG. Further proteins with altered expression in the patient included fibrinogen, TTH and serum amyloid P, which have all been highlighted in previous biomarker studies in PD. Long term monitoring revealed that both chronic inflammation and oxidative stress rose over time as progression of neurodegenerative symptoms worsened in the patient. Further studies are needed to
confirm the prevalence of the systemic disruptions and disease burden noted in this patient in other patients with NBIA.

DFP therapy was well tolerated with limited impact on systemic iron trafficking. It provided a reduction in chronic inflammation in the patient. Antioxidant therapy was similarly well tolerated and was able to reduce chronic inflammation and mitigate increases in oxidative stress in the patient. ALA and combination therapy with ALA and NAC provided this reduction in the systemic disease burden, but treatment with NAC alone provided no therapeutic benefit. Both of these therapies mitigated the systemic disease burden in PLAN, but it did not have a significant impact on the disease severity or progression in the patient. Two previous studies were highlighted which demonstrated that early intervention may be key to improving the efficacy of both chelation and antioxidant therapies. However, even in advanced cases these therapies may be efficacious in limiting the systemic disease burden of neurodegeneration in PLAN. Despite the lack of efficacy in improving motor symptoms the biomarker investigation in this study highlights a systemic disease burden in PLAN, which was targeted by therapy. Additionally, novel candidate biomarkers, fibrinogen, transthyretin, adiponectin, zinc alpha 2-glycoprotein and serum amyloid P are proposed.
References


Inflammation & Allergy Drug Discovery, 3(1), 73–80.


neurodegeneration (PKAN) treated with deferiprone: Results of a phase II pilot trial. Movement Disorders, 26(9), 1755–1759.

5.1. Abstract

Pantothenate Kinase-Associated Neurodegeneration (PKAN) is a rare, recessive monogenic disorder characterized by iron accumulation and degeneration within the BG which is accompanied by the rapid and progressive onset of debilitating motor symptoms. Limitations in the understanding of the systemic pathophysiology of PKAN have prevented the pursuit of systemic blood-based biomarkers of disease; a valuable clinical tool in assessing disease onset, severity and progression as well as in measuring the efficacy of therapy. To address this limitation, a first of its kind study investigation into a large panel of candidate blood-based biomarkers was performed in a cohort of 30 patients with PKAN being treated through the UCSF Benioff Children’s Hospital in Oakland California. The patients’ systemic iron levels were largely normal, but a significant reduction in levels of transferrin was observed. Elevations of CRP (460%) and the oxidative stress marker malondialdehyde (MDA, 40%) provide evidence for a systemic disease burden of chronic inflammation and oxidative stress in PKAN. Proteomic analysis revealed a series of possible candidate blood-based biomarkers that were abnormally expressed in PKAN including: fibrinogen (60% increased), transthyretin (TTH, 35% decreased), adiponectin (50% increased), zinc alpha 2-glycoprotein (ZAG, 20% decreased) and retinol binding protein (RBP, 20% decreased). ZAG and RBP may be indicative of systemic reductions in retinol and thyroid hormone. Expression of all these markers significantly correlated with disease severity in the patients as measured by R2* MRI or the Barry-Albright Dystonia (BAD) Scale or both. Notably, both fibrinogen and TTH correlated with disease severity in patients. In severely affected patients both fibrinogen and TTH were expressed at levels outside of the control range. Additionally, reductions in ZAG and RBP may be present early in the disease progression in less severely affected patients.
These markers were further assessed as the patients with PKAN underwent an randomized, double-blinded, placebo-controlled phase 3 clinical trial of the iron chelator deferiprone (DFP). DFP was well tolerated in patients and showed a high degree of efficacy in lowering brain iron accumulation; a reduction in R2* MRI (35%) was seen in all treated patients, which was significantly greater than what was observed in the placebo patients; who largely did not show a reduction in iron levels. DFP had minimal impact on systemic iron levels, but it did normalize transferrin expression. It also demonstrated efficacy in mitigating the systemic disease burden by lowering CRP and MDA levels in patients. Some evidence was found for DFP slowing the progression of motor symptoms. In 6 of the patients a decrease (2/6) or no change in BAD score (4/6) was seen. The mean increase in BAD score during the trial was lower in treated patients, but not significantly so. Due to its high efficacy in removing brain iron accumulation as well as its potential to mitigate the systemic disease burden, DFP appears to be a promising tool in the treatment of PKAN.

5.2. Introduction

Recent advances in genetic sequencing and brain imaging techniques have enabled the delineation of a novel group of rare early-onset, familial, monogenic neurodegenerative disorders; primarily targeting the BG, that are characterized by extrapyramidal symptoms as well as cognitive and motor dysfunction. This group of disorders is known as neurodegeneration with brain iron accumulation (NBIA). These disorders are named after their shared hallmark of excess iron accumulation within the BG. The degeneration and iron accumulation that occurs in NBIA primarily targets the globus pallidus (GP) and substantia Nigra (SN). Pantothenate kinase associated neurodegeneration (PKAN) is the most common form of NBIA and was the first NBIA disorder to have its underlying genetic cause delineated (Zhou et al. 2001). It is caused by mutations in PANK2, the gene encoding an isoform of pantothenate kinase, which is responsible for catalyzing the conversion of pantothenate into 4-phosphopantothenate; the first step in the production of Co-enzyme A (CoA) (Zhou et al., 2001). The specific isoform mutated in PKAN; pantothenate kinase-2 (PANK2), shows a ubiquitous expression
pattern throughout the body, but is more dominantly expressed in both the retina and BG than other isoforms of pantothenate kinase (Zhou et al. 2001). CoA is involved in numerous metabolic pathways including the citric acid cycle and fatty acid metabolism as well as cholesterol and sphingolipid synthesis (Rouault, 2013). Defects in PANK2 have the potential to have widespread effects ranging from mitochondrial dysfunction to impaired membrane synthesis.

Though PKAN and other NBIA disorders are relatively rare, with incidences in the range of ~1:1,000,000 (Hogarth, 2015), the presentation of extrapyramidal symptoms as well as degeneration and iron accumulation inside of the BG strongly parallels other more common neurodegenerative disorders such as Parkinson’s disease (PD). Thus, research efforts in PKAN provide insight into these shared hallmarks of disease pathophysiology. Research into better understanding of the underlying mechanisms which lead to dysfunction in NBIA not only benefits the relatively small group of individuals affected by these disorders, but also the larger patient population which is affected by PD.

PKAN can be divided into an early-onset and rapidly progressing classic form as well as a later-onset and more slowly progressive atypical form (Hayflick et al., 2003), though in reality many cases exist on a spectrum between these two groupings. The classical presentation of PKAN represents the majority of cases. It typically occurs in early childhood with an onset prior to 6 years of age and rapid disease progression (Gregory, Polster, & Hayflick, 2009; Hayflick et al., 2003). For atypical PKAN the age of onset is later; around 13-14 years of age and the disease progression is slower (Gregory et al., 2009).

5.2.1. PKAN Pathology

The pathology of PKAN is predominantly within the central nervous system (CNS) and is focused in the BG. Both iron accumulation and degeneration targeted primarily in the GP are hallmarks of PKAN; targeting of the SN is also frequently seen. Histopathological investigations by Krueger et al. (2011) revealed the formation of abnormal spheroid structures that formed in associated with degenerating neurons as well as extensive iron deposits within the GP.
5.2.2. Systemic Investigations of PKAN

Despite the ongoing delineation of the symptomology and neuropathophysiology of PKAN research into the systemic pathophysiology of PKAN is limited. To date, preliminary investigations into the systemic state of PKAN using animal models (Kuo et al., 2005), primary cell cultures (Campanella et al., 2012; Santambrogio et al., 2015) as well as metabolic profiling (Leoni et al., 2012), have been performed, but questions remain surrounding the systemic impact of degeneration inside the central nervous system (CNS). Improving understanding of the systemic dysfunction which occurs in PKAN helps to characterize systemic processes that may be occurring and contributing to disease progression in patients. It also provides the opportunity to assess the systemic disease burden of inflammation and oxidative stress in patients. Finally, these investigations give the opportunity to explore and evaluate candidate systemic biomarkers, which are currently lacking in NBIA. Systemic blood-based biomarkers are comparably easier to access and lower in cost to measure; this also includes both lower training costs and financial costs, than existing clinical tools in NBIA. Furthermore limitations exist in the available clinical tools in NBIA, such as genetic analyses, MRI and PET scans as well clinical rating scales. These are primarily due to the expense or training required in their administration. Ultimately, systemic blood-based biomarkers are well suited to complement these existing methods and provide additional utility in the assessment of NBIA. The use of these biomarkers is predicated on a greater understanding of the systemic disease state in PKAN and other NBIA disorders however.

A number of hallmarks of neurodegeneration within the CNS in NBIA patients remain poorly explored or unexplored systemically. A key area of interest in NBIA is the state of systemic iron metabolism. Numerous questions remain unanswered including: how systemic iron handling is affected, what contribution this ultimately has to the trafficking of iron into the brain and if there are signs of systemic changes in iron trafficking that could be targeted to limit brain iron accumulation. Furthermore, a disease burden including oxidative stress and elevated inflammation is often noted in the CNS in NBIA and PD (Adibhatla & Hatcher, 2010; Mariani, Polidori, Cherubini, & Mecocci, 2005;
Nagatsu & Sawada, 2005; Niranjan, 2014; Núñez et al., 2012). Preliminary studies in primary cell cultures have demonstrated the potential for elevated oxidative stress due to ROS in systemic tissues in NBIA (Campanella et al., 2012; Santambrogio et al., 2015). Translational studies confirming the presence of systemic oxidative stress and exploring the systemic inflammatory state in PKAN patients still need to be performed. One hallmark of NBIA that has been explored systemically is dysfunctional lipid metabolism. The work of Leoni et al. (2012) demonstrated global defects in cholesterol, lipid and bile metabolism in PKAN; suggesting that defects in CoA production due to PANK2 mutations may impact the downstream synthesis of lipids reliant on CoA precursors. In addition to these areas recent studies in PD have also highlighted a number of potential candidate systemic markers that may unique markers in patients affected by neurodegeneration (Andican et al., 2012; Chahine, Stern, & Chen-Plotkin, 2014; Chen et al., 2009; Chen, O’Reilly, Schwarzschild, & Ascherio, 2007; Saracchi, Fermi, & Brighina, 2014; Wong et al., 2010), which could be explored in NBIA. Collectively these areas and the candidate markers recently proposed in PD represent promising targets for blood-based systemic biomarkers in PKAN.

5.2.3. Chelation

As outlined in Chapter 4, chelation therapy is an emerging therapeutic option in NBIA disorders such as PKAN. The goal of this therapy is the targeted removal of iron accumulating inside of the BG in order to limit its potential to contribute oxidative tissue damage. For a complete overview of chelation therapy in NBIA see Chapter 4.

5.2.4. Overview

This study is the first large, multi-phase investigation into novel blood-based biomarkers in NBIA. This study is being conducted as an investigation of a cohort of 30 patients with PKAN. The focus of the first part of this study is the identification of potential systemic biomarkers of NBIA which are explanatory of the systemic disease state in NBIA. In the second part, these markers will then be explored as markers of the pharmacological response to therapy with the iron chelator DFP. Samples will be collected from these
patients as they participate in the ongoing phase 3 clinical trial of DFP treatment targeting brain iron accumulation in PKAN. This provides the opportunity to evaluate candidate biomarkers measured in the first part of this study as possible indicators of the pharmacological response to therapy. Collectively the two parts of this study will provide novel insight into systemic dysfunction in PKAN and a characterization of the state of systemic iron metabolism in this disorder. Additionally, it will assess the impact of an emerging therapy in NBIA in patients with PKAN.

5.3. Methods

5.3.1. Study Setup

PKAN Patient Group

30 patients with a genetically confirmed diagnosis of PKAN were recruited at the UCSF Benioff Children’s Hospital in Oakland California as a substudy of two on going studies into DFP therapy (ClinicalTrials.gov Identifier: NCT01741532, Study ID: TIRCON2012V1 and ClinicalTrials.gov Identifier: NCT02174848, Study ID: TIRCON2012V1-EXT). The study was approved by the Hospital’s Research Ethics Board and all patients provided informed consent to participate in the study. Minors under the age of 17 also had informed consent provided by their parents or guardians. For full patient demographics see (Table 5-1).

Case Study of a Patient with Phospholipase A2G6 Associated Neurodegeneration (PLAN)

Results from a case study investigation of a single patient with an alternate form of NBIA, Phospholipase A2G6-Associated Neurodegeneration (PLAN) are also presented where applicable. This patient was treated for 37 weeks with DFP. All biomarker measurements were performed in this patient as previously outlined. For full PLAN patient background and details of the biomarker analyses in the patient see Chapter 4.

5.3.2. Study Controls

16 control patients were recruited at the University of Victoria in Victoria, B.C. The participation of controls was jointly approved by the University of Victoria and the
Vancouver Island Health Authority research ethics boards (CREB #J2013-009). All controls provided informed consent to participate in this study. Minors under the age of 17 also had informed consent provided by their parents or guardians. For full control demographics see Table 5-1. A single control with a previous history of anorexia nervosa was removed from the control group for a final control group of 15 patients. See Appendix A for further details on this control.
Table 5-1. Demographic Characteristics of Control Group in comparison to study groups of patients with Pantothenate Kinase Associated Neurodegeneration (PKAN).

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 15)</th>
<th>PKAN Baseline Main (n =15)</th>
<th>PKAN Baseline Proteomics (n =16)</th>
<th>PKAN 18 Months of Deferiprone Therapy (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>21.5 + 1.59</td>
<td>16.93 + 3.02</td>
<td>16.12 + 2.85</td>
<td>20.93 + 2.3</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169.9+ 3.45</td>
<td>144 + 7.37</td>
<td>144.2 + 7.13</td>
<td>157.9 + 6.09</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.52 + 4.8</td>
<td>40.2 + 4.48</td>
<td>39.76 + 4.2</td>
<td>55.29 + 5.71</td>
</tr>
<tr>
<td>Sex</td>
<td>7 Male 8 Female</td>
<td>7 Male 8 Female</td>
<td>7 Male 9 Female</td>
<td>9 Male 6 Female</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>13 Caucasian 2 Asian</td>
<td>8 Caucasian 2 Asian 4 Hispanic 1 African American</td>
<td>8 Caucasian 2 Asian 5 Hispanic 1 African American</td>
<td>13 Caucasian 3 Hispanic</td>
</tr>
<tr>
<td>BMI</td>
<td>23.19 + 1.33</td>
<td>18.34 + 0.76</td>
<td>18.03 + 0.71</td>
<td>33.75 + 2.57</td>
</tr>
<tr>
<td>Age at Diagnosis</td>
<td>N/A</td>
<td>14.4 + 2.89</td>
<td>13.82 + 2.77</td>
<td>14.41 + 2.36</td>
</tr>
<tr>
<td>Days Since Diagnosis</td>
<td>N/A</td>
<td>999.7 + 334.93</td>
<td>871.2 + 292.5</td>
<td>2328 + 409.35</td>
</tr>
<tr>
<td>PedsQL&lt;sup&gt;TM&lt;/sup&gt;- Physical Score</td>
<td>N/A</td>
<td>44.72 + 5.949</td>
<td>44.47 + 5.50</td>
<td>35.63 + 6.19</td>
</tr>
<tr>
<td>PedsQL&lt;sup&gt;TM&lt;/sup&gt;- Psychosocial Score</td>
<td>N/A</td>
<td>76.85 + 3.49</td>
<td>74.65 + 2.85</td>
<td>72.15 + 4.7</td>
</tr>
<tr>
<td>PedsQL&lt;sup&gt;TM&lt;/sup&gt;-Total Score</td>
<td>N/A</td>
<td>65.54+3.4</td>
<td>64.09+3.18</td>
<td>58.73 + 4.71</td>
</tr>
<tr>
<td>Globus Pallidus Iron Deposition (R2* MRI)</td>
<td>N/A</td>
<td>78.7 + 10.15 (n = 10)</td>
<td>76.5 + 8 (n = 8)</td>
<td>63.78 + 7.74 (n = 9)</td>
</tr>
<tr>
<td>Severity of Motor Symptoms (BAD Scale)</td>
<td>N/A</td>
<td>15.67 + 2.48</td>
<td>15.88 + 2.25</td>
<td>20.67 + 1.84</td>
</tr>
</tbody>
</table>
5.3.3. **Deferiprone Treatment**

The Phase III clinical trial of DFP treatment in PKAN patients was a randomized, double-blinded and placebo-controlled study. Patients were randomly assigned to either the treatment (2/3 of patients) or placebo (1/3 of patients) groups. Deferiprone 80 mg/mL oral solution was administered twice daily for 18 months to treated patients. An initial dose of 5 mg/kg/day was administered for 6 weeks. Dosage was then increased to 10mg/kg bidaily and finally to 15 mg/kg bidaily. A matching placebo oral solution was given to placebo patients twice daily for 18 months. DFP was graciously provided for this trial by ApoPharma Inc.

5.3.4. **Blood Sample Collection and Processing**

The main study was setup to assess patients and collects samples at baseline, 6 months, 12 months and 18 months of therapy. Upon initiation of the sub-study additional blood samples were collected at each of these pre-set time points. Notably, some time collection points had elapsed prior to the initiation of our substudy. Samples from 24 collection dates that occurred prior to the initiation of the sub-study were acquired from the TIRCON study biobank at the Institute for Medical Statistics and Epidemiology at the Technical University in Munich, Germany. These samples were prioritized for inclusion in the proteomic analyses. See Appendix O for additional details of sample collection and timepoints. For assessment of therapy in the analyses presented below the 18 months time point is highlighted.

After all patients had completed 18 months of therapy the blinding for the study was lifted. Due to the initiation of the study after the main study had already started placebo patients were considered at baseline at their first time point in the study. An untreated placebo patient that had its first sample available 6 months in the study would be considered at baseline for the purpose of our analysis. All patient timepoints noted here are based on the unblinded adjusted values.

All blood samples were collected by a trained phlebotomist. All baseline samples were from patients naïve to deferiprone treatment. Sample collection was performed at
UCSF Benioff Children’s Hospital in Oakland California. Samples for plasma analysis were collected in heparinized tubes and spun for 10 min at 1000 g. The plasma layer was removed and aliquoted. Plasma samples were frozen in cryovials and stored at -80 °C. Serum samples were collected in untreated serum tubes and allowed to sit for 40 min before being spun for 10 min at 1000 g. The serum layer was removed and aliquoted for storage in cryovials at -80 °C until analysis.

5.3.5. **Patient Monitoring**

Additional blood samples were collected for ongoing patient monitoring during the trial. This assessment included the measurement of a complete blood cell count as well as amylase, aspartate transaminase, blood urea nitrogen (BUN), creatinine, total protein, aminotransferase (ALT), lactate dehydrogenase (LDH) and serum ferritin (See Table for Full Marker details). These blood samples were collected by a trained phlebotomist. All blood chemistry markers were measured by hospital laboratory services at UCSF Benioff Children’s Hospital. These blood chemistry markers were measured in patients at baseline. Additionally, weekly monitoring of these markers occurred over the course of deferiprone therapy. The monitoring time points which corresponding to the collected 6-month, 12-month and 18-month timepoints were including in the analysis.

5.3.6. **Disease Severity Measurements**

*Magnetic Resonance Imaging*

Blinded R2* MRI of the amount of iron accumulation inside the GP was performed on a selection of patients at baseline at UCSF Benioff Children’s Hospital. Patients who could not comfortably remain still for the MRI or who were undergoing deep brain stimulation did not receive MRIs. MRI analysis was performed blinded to patient treatment status at Newcastle University, UK.

*Barry-Albright Dystonia (BAD) Scale*

Each patient was recorded for the purpose of assessment using the BAD scale at UCSF Benioff Children’s Hospital at baseline, 6 months, 12 months and 18 months.
Independent blinded assessment of each patient video using the BAD scale was performed to score the severity of motor symptoms of dystonia in each patient at the Oregon Health & Science University in Portland, Oregon.

*Pediatric Quality of Life Inventory™*

The pediatric quality of life inventory (PedsQL™) was completed by patients at baseline and 18 months at the UCSF Benioff Children’s Hospital. Additionally, for children younger than 18 enrolled in the study a parental proxy report was also completed. Adults enrolled in the study completed the adult version of the PedsQL™. For individual patients who were unable to complete the PedsQL™ independently a parental proxy report was used in place of their score (~19/30 patients). An assessment of the quality of life in a given area improves the score in that category increases. Thus, a decreasing score corresponds to decreasing quality of life and an increasing score corresponds to increasing quality of life.

5.3.7. *Biomarker Measurements*

All biomarker measurements were performed blinded to the treatment status of the patient. In order to limit intraassay variability each sample plate was run with an internal standard curve. Additionally, each plate was run with a mixture of control and patient samples. The biomarker panel that is detailed in Chapter 4 was measured in the patients with PKAN. All biomarker measurements were performed as outlined in Chapter 4.

5.3.8. *Data Analyses*

Statistical analyses were conducted using R (R Core Team, 2016). Data was initially plotted and normality was tested based on outliers, ranges and distributions. Summary statistics including mean, SD, SE and 95% confidence intervals were then calculated for the data. Due to the missing early sample time points PKAN patients were compared to controls grouped by study timepoint and not longitudinally. A secondary analysis of PKAN patients at baseline to PKAN patients following 18 months of deferiprone treatment was also performed. Due to the lack of independence between these two groups these
analyses were performed independently using a 2-tailed student’s t-test for normally distributed data and using the Mann-Whitney-Wilcoxon U test for non-normal, skewed data. Due to differences between control and patient populations in baseline demographics further analysis was done by ANCOVA to evaluate the impact of ethnicity, sex, height, weight and age on the above performed tests. Each demographic factor was tested visually and by correlation to ensure that it met the base assumptions to operate an ANCOVA using the R packages “lattice” (Deepayan Sarkar, 2008), “car” (Fox & Weisberg, 2011), “heplots” (Friendly, 2007) and “effects” (Fox, 2003). Following these tests a step-wise ANCOVA model was performed for each marker. The adjusted p-values from the final model are presented for each factor. For markers that were significantly different between the control and PKAN patients at baseline Pearson correlations were performed to determine the correlation between these markers and the markers of patient disease severity: R2*MRI, BAD, and PedsQL™. Results were presented graphically using the R packages “ggplot2” (Wickham, 2009) and “gridExtra” (Auguie, 2016).

5.4. Results

5.4.1. PKAN Patients at Baseline

See Appendix P for full PKAN baseline biomarker results.

Iron Metabolism

No significant difference was seen in the systemic iron levels in the PKAN patients at baseline (Table 5-2). There was no significant difference in the serum iron levels, TIBC and transferrin saturation. Plasma transferrin levels were significantly reduced in the PKAN patients at baseline (Figure 5-1A), p = 0.044. Plasma alpha hemoglobin (Figure 5-1B) and sTfR expression were slightly, but not significantly elevated. Additionally, patient serum ferritin levels were in the lower end of the normal range at baseline.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Control (n = 15)</th>
<th>PKAN Baseline (n = 15)</th>
<th>PKAN 18 Months of Deferiprone Therapy (n = 15)</th>
<th>Significance Control vs Baseline</th>
<th>Significance Control vs 18 Months</th>
<th>Baseline vs 18 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Ceruloplasmin** (fmol/uL)</td>
<td>1104 ± 64.62</td>
<td>1193 ± 96.21</td>
<td>1368 ± 127.92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Haptoglobin** (fmol/uL)</td>
<td>15540 ± 1905.56</td>
<td>13980 ± 2915.48</td>
<td>16500 ± 3264.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Hemopexin** (fmol/uL)</td>
<td>8280 ± 285.35</td>
<td>8418 ± 334.04</td>
<td>8829 ± 414.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Transferrin** (fmol/uL)</td>
<td>17150 ± 762.91</td>
<td>15170 ± 543.39</td>
<td>17260 ± 579.38</td>
<td>0.044</td>
<td></td>
<td>0.013</td>
</tr>
<tr>
<td>Plasma Hemoglobin Alpha Chain** (Normalized Peak Area Ratio)</td>
<td>7.29 ± 0.69 x10^{-3}</td>
<td>14.2 ± 3.7 x10^{-3}</td>
<td>16.2 ± 1.9 x10^{-3}</td>
<td>0.086</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>Plasma Soluble Transferrin Receptor** (mg/L)</td>
<td>2.84 ± 0.35</td>
<td>3.737 ± 0.51</td>
<td>3.877 ± 0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Iron (µmol/L)</td>
<td>13.67 ± 1.76</td>
<td>14.15 ± 2.79</td>
<td>14.05 ± 1.74</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Iron Binding Capacity (µmol/L)</td>
<td>55.72 ± 2.56</td>
<td>53.86 ± 2.15</td>
<td>55.68 ± 2.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin Saturation (%)</td>
<td>25.10 ± 3.83</td>
<td>25.58 ± 3.99</td>
<td>26.2 ± 3.57</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>Reference Range</th>
<th>PKAN Baseline</th>
<th>PKAN 18 Months of Deferiprone Therapy</th>
<th>PKAN Baseline</th>
<th>PKAN 18 Months of Deferiprone Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Hemoglobin (g/L)</td>
<td>123-157</td>
<td>134.1 ± 0.59</td>
<td>141.3 ± 2.42</td>
<td>Within Reference Range</td>
<td>Within Reference Range</td>
</tr>
<tr>
<td>Serum Ferritin (µg/L)</td>
<td>15-207</td>
<td>37.21 ± 6.32</td>
<td>28.73 ± 7.71</td>
<td>Within Reference Range</td>
<td>Within Reference Range</td>
</tr>
</tbody>
</table>

*All values are ± SE

**For baseline measurements of plasma proteins n = 16
Figure 5-1. Plasma expression of the iron trafficking protein transferrin in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN) at baseline and after 18 months of deferiprone (DFP) therapy (n = 16 at baseline and n = 15 at 18 months) relative to controls (n = 15); as measured by multiple reaction monitoring mass spectrometry proteomics at the University of Victoria Genome B.C. Proteomic Center. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma transferrin levels were significantly reduced in patients at baseline p = 0.04 (*, 2-tailed, unpaired, students t-test). A significant elevation in transferrin levels was seen following 18 months of deferiprone treatment P = 0.13 (*, 2-tailed, unpaired, students t-test).

Systemic Disease Burden

Signs of a systemic inflammatory burden were seen in the PKAN patients at baseline (Table 5-3). CRP levels were significantly elevated at baseline compared to controls (Figure 5-2A), p = 0.01. Additionally, elevated plasma levels of the inflammatory cytokines IL-6 (Figure 5-2B), IL-10 (Figure 5-2C) and TNFα (Figure 5-2D) were seen in a subset of the patients with PKAN. Increased systemic oxidative stress was also seen in the patients at baseline. The plasma levels of the antioxidant enzyme glutathione peroxidase were significantly reduced at baseline (Figure 5-3A), p = 0.02. This was accompanied by a significant increase in the oxidative stress marker MDA in both the total MDA pool (Figure 5-3B) and the free MDA pool (Figure 5-3C), p = 0.044 and p = 0.01.
**Table 5-3.** Systemic oxidative and inflammatory disease burden of patients with Pantothenate Kinase-Associated Neurodegeneration (PKAN) at baseline and following 18 months of deferiprone therapy*.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control (n = 15)</th>
<th>PKAN Baseline (n = 15)</th>
<th>PKAN 18 Months of Deferiprone Therapy (n = 15)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Normalized Peak Area Ratio)</td>
<td>0.098 ± 0.054</td>
<td>0.014 ± 0.0037</td>
<td>0.016 ± 0.0019</td>
<td>0.014</td>
</tr>
<tr>
<td>Malondialdehyde (µM)</td>
<td>2.27 ± 0.27</td>
<td>3.17 ± 0.34</td>
<td>2.99 ± 0.36</td>
<td>0.047</td>
</tr>
<tr>
<td>Free Malondialdehyde (µM)</td>
<td>0.45 ± 0.05</td>
<td>0.74 ± 0.093</td>
<td>0.52 ± 0.11</td>
<td>0.015</td>
</tr>
<tr>
<td>Glutathione Peroxidase 3 (fmol/µL)</td>
<td>92.95 ± 4.51</td>
<td>79.18 ± 5.5</td>
<td>82.59 ± 4.39</td>
<td>0.021</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>2.68 ± 0.28</td>
<td>3.36 ± 0.22</td>
<td>0.012</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.84 ± 0.22</td>
<td>3.03 ± 0.70</td>
<td>3.2 ± 0.66</td>
<td>0.068</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.33 ± 0.36</td>
<td>3.36 ± 0.98</td>
<td>4.71 ± 0.99</td>
<td>0.037</td>
</tr>
</tbody>
</table>

*All values are ± SE

**For baseline measurements of plasma proteins n = 16
Figure 5-2. Plasma expression of the acute inflammatory protein CRP (A), and the inflammatory cytokines IL-6 (B), IL-10 (C) and TNFα (D) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN) at baseline and following 18 months of deferiprone (DFP) therapy (n = 16 for CRP and n = 15 for cytokines at baseline, n = 15 at 18 months) relative to controls (n = 15); as measured by SISCAPA immunoassay enhanced multiple reaction monitoring mass spectrometry proteomics (A) and ELISA (B-D). Plasma CRP levels in PKAN patients were significantly elevated at baseline \( p = 0.13 \), but not following 18 months of deferiprone therapy (*, 2-tailed, unpaired, students t-test). No significant difference was seen in the levels of the inflammatory cytokines IL-6, IL-10 and TNFα at baseline compared to controls. There was a significant difference in the levels of IL-10, \( p = 0.012 \), and TNFα, \( p = 0.037 \), in patients compared to controls following 18 months of DFP therapy (*, 2-tailed, unpaired, students t-test).
Figure 5-3. Plasma expression of the antioxidant enzyme glutathione peroxidase (A), and the lipid peroxidation products malondialdehyde (MDA, B) and free MDA (C) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN) at baseline and following 18 months of deferiprone (DFP) therapy (n = 16 for glutathione peroxidase and n = 15 for MDA and free MDA at baseline, n = 15 at 18 months) relative to controls (n = 15 for glutathione peroxidase and MDA and n = 9 for free MDA); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics (A) and using a NMPI based colourimetric assay (B and C). Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma glutathione peroxidase levels were significantly reduced in patients compared to controls at baseline, p = 0.02, but not following 18 months of treatment with deferiprone (*, 2-tailed, unpaired, students t-test). Plasma MDA and free MDA levels were significantly elevated in patients at baseline, p = 0.046 and p = 0.01, but not following 18 months of treatment with DFP (*, 2-tailed, unpaired, students t-test).

Lipid Metabolism

Disruptions in the expression pattern of a number of proteins associated with lipid metabolism were noted in the PKAN patients at baseline (Supplementary Table 0-10). Levels of adiponectin were significantly elevated (Figure 5-4B), p = 0.011. This elevation was also seen in an investigation of a single patient with PLAN. Conversely levels of zinc alpha 2-glycoprotein were significantly reduced in PKAN (Figure 5-4D), p = 0.04. The levels of phospholipid transfer protein were also significantly reduced in patients (Figure 5-4C),
$p = 0.033$. Additionally, the mean levels of the numerous apolipoproteins including apolipoprotein AI, AIV, CI-IV, D, E and F were all lower in the patients with PKAN (Supplementary Table 0-10).

**Figure 5-4.** Plasma expression of retinol-binding protein (A), and the lipid metabolism associated proteins zinc alpha 2-glycoprotein (B), phospholipid transfer protein (C) and adiponectin (D) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16) relative to controls (n = 15); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma retinol-binding protein, zinc alpha 2-glycoprotein and phospholipid transfer protein levels were significantly reduced in patients, $p = 0.02$, 0.04 and $p = 0.03$ (2-tailed, unpaired, students t-test). Plasma adiponectin levels were significantly elevated in patients, $p = 0.01$ (2-tailed, unpaired, students t-test). This elevation in adiponectin was also seen in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN).
Candidate Biomarkers of Interest

Baseline proteomic analysis revealed a number of other candidate proteins of interest which were significantly different at baseline than the control group in the patients with PKAN (Supplementary Table 0-10). The plasma levels of retinol-binding protein (RBP) were significantly reduced in patients at baseline (Figure 5-4A), p = 0.02. A large increase was seen in the plasma expression of the alpha, beta and gamma chains of fibrinogen (Figure 5-5A and Supplementary Table 0-10), p<0.001, p<0.001 and p<0.001. Finally, the carrier protein transthyretin (TTH) was significantly lower in the plasma of patients with PKAN (Figure 5-5B), p = 0.06.

![Figure 5-5. Plasma expression of fibrinogen alpha chain (A), and transthyretin (B) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16) relative to controls (n = 15); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma fibrinogen alpha chain levels were significantly elevated in patients, p < 0.001 (2-tailed, unpaired, students t-test). Plasma transthyretin levels were significantly reduced in patients p<0.001 and p = 0.006 (2-tailed, unpaired, students t-test). This elevation of fibrinogen alpha chain was also seen in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN). Plasma transthyretin levels were significantly reduced in patients at baseline p = 0.006 (2-tailed, unpaired, students t-test). This reduction in transthyretin were also seen in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN).](image)

**Disease Correlations**

**Globus Pallidus Iron Loading**

The intensity of iron loading in the GP, as measured by R2* MRI, was correlated to the expression levels of zinc alpha 2-glycoprotein, retinol-binding protein and fibrinogen alpha chain. Significant positive correlations were seen between R2* MRI and zinc alpha...
2-glycoprotein (Figure 5-6A), \( p = 0.03 \) \( R = 0.75 \), retinol-binding protein (Figure 5-6B), \( p = 0.04 \) \( R = 0.72 \), and fibrinogen alpha chain (Figure 5-6C) \( p = 0.03 \) and \( R = 0.75 \). The beta and gamma chains of fibrinogen showed similar correlations to GP iron loading (Data not shown).

Figure 5-6. Correlations between globus pallidus iron accumulation as measured by R2* MRI and the plasma proteins zinc alpha 2-glycoprotein (A), retinol-binding protein (B) and fibrinogen alpha chain (C); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, \( n = 8 \)). A significant positive correlation between the accumulation of iron in the globus pallidus and zinc alpha 2-glycoprotein, \( p = 0.03 \), retinol-binding protein, \( p = 0.04 \), and fibrinogen alpha chain \( p = 0.03 \) was observed in PKAN patients (Pearson’s correlation).
Severity of Motor Symptoms

In addition to the correlations seen to brain iron accumulation in the GP, correlations were between a series of the systemic biomarkers and the severity of motor symptoms of dystonia as measured by the BAD scale. A significant positive correlation was seen between the expression levels of fibrinogen alpha chain (Figure 5-7A), $p = 0.03$ $R = 0.54$, as well as zinc alpha-2 glycoprotein (Figure 5-7C), $p = 0.008$ $R = 0.63$, and the severity of motor symptoms. Conversely, a significant negative correlation was seen between plasma transthyretin expression (Figure 5-7B), $p = 0.009$ $R = -0.63$, and the severity of motor symptoms. Additionally, positive correlations were seen between glutathione peroxidase levels (Figure 5-8A), $p = 0.02$ $R = 0.58$, as well as plasma CRP levels (Figure 5-8B), $p = 0.05$, $R = 0.50$. The beta and gamma chains of fibrinogen showed similar correlations to disease severity (Data not shown).
Figure 5-7. Correlations between the severity of motor symptoms as measured by the Barry-Albright Dystonia (BAD) scale and fibrinogen alpha chain (A), transthyretin (B) and zinc alpha 2-glycoprotein (C); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16). A significant positive correlation between the severity of motor symptoms and zinc alpha 2-glycoprotein, $p = 0.008$, and fibrinogen alpha chain $p = 0.03$ was observed in PKAN patients (Pearson's correlation). A significant negative correlation between the severity of motor symptoms and zinc alpha 2-glycoprotein, $p = 0.008$, and fibrinogen alpha chain $p = 0.009$ was observed in PKAN patients (Pearson's correlation).
Figure 5-8. Correlations between the severity of motor symptoms as measured by the Barry-Albright Dystonia (BAD) scale and the plasma levels of glutathione peroxidase (A) and CRP (B); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics (A) and SISCAPA immunoassay enhanced multiple reaction monitoring mass spectrometry proteomics (B) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16). A significant positive correlation between the severity of motor symptoms and glutathione peroxidase, *p* = 0.02, and nearly significant correlation with CRP, *p* = 0.05, was observed in PKAN patients (Pearson’s correlation).

Strong interrelationships were seen in the patients with PKAN at baseline between these markers which correlated with measures of disease severity in patients with PKAN: GP iron accumulation and the severity of motor symptoms. The levels of fibrinogen alpha chain had a significant negative correlation with transthyretin expression (Supplementary Figure 0-13A), *p* = 0.049, *R* = -0.49. Plasma transthyretin expression also negatively correlated with the levels of zinc alpha 2-glycoprotein (Supplementary Figure 0-13C), *p* = 0.1 *R* = 0.42. Plasma expression of zinc alpha 2-glycoprotein positively correlated with fibrinogen alpha chain (Supplementary Figure 0-13B), *p* = 0.003 *R* = 0.69, and retinol binding-protein (Supplementary Figure 0-13D), *p* < 0.001 *R* = 0.81. Notably the expression ranges of these proteins in the PKAN patients displayed a partial overlap with the levels seen in the control group. A 3-factor analysis showed that reductions in transthyretin and elevations in fibrinogen alpha chain were seen in a unique patient group, which had the most severe motor symptoms of dystonia (Figure 5-9). This trend was also seen in a single patient with PLAN.
Figure 5-9. A 3-factor representation of plasma expression levels of fibrinogen alpha chain and transthyretin in comparison to disease severity of patients with Pantothenate Kinase-Associated Neurodegeneration (PKAN, n = 16), a single patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN, n = 1) and a control group (n = 15). Plasma proteins were measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics. Control patients were set to a disease severity of 0 and a mild-moderate UPDRS score in the PLAN patient was converted to a BAD score of 20. The most severely affected patients, based on BAD or UPDRS scores represent a unique group which is distinctly separated from the control patients by elevated fibrinogen alpha chain levels and reduced transthyretin levels.

5.4.2. Outcome of Deferiprone Therapy

See Appendix P for a highlight of PKAN DFP trial biomarker results.

Monitoring

Based on the monitoring panel no significant adverse reactions were noted during 18 months of DFP treatment (Data not shown). Plasma levels of the liver health marker ALT and plasma expression of LDH remained within the normal range at the 6-month, 12-month and 18-month timepoints of DFP therapy. (Data not shown).
**Disease Severity**

DFP treatment lowered brain iron accumulation in the GP of treated patients. A significant decrease was seen in the levels of GP iron accumulation in treated, but not in placebo patients, \( p<0.001 \) (Table 5-4). BAD scores rose less over 18 months for patients who were treated with DFP than patients who were in the placebo group. No significant trends were noted in the PedsQL™ assessment in treated patients in comparison to untreated patients.

**Table 5-4.** Measurements of disease severity in patients with Pantothenate Kinase-Associated Neurodegeneration (PKAN) at baseline and following 18 months of deferiprone therapy*.

<table>
<thead>
<tr>
<th>Marker</th>
<th>PKAN All Baseline (n=15)</th>
<th>PKAN Placebo Baseline (n=15)</th>
<th>PKAN Placebo 18 Months (n=15)</th>
<th>Placebo Change</th>
<th>PKAN Deferiprone Baseline (n=15)</th>
<th>PKAN Deferiprone 18 Months (n=15)</th>
<th>Deferiprone Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI Globus Pallidus (R2*)</td>
<td>90.23 ± 7.76</td>
<td>81.39 ± 12.81</td>
<td>83.39 ± 12.84</td>
<td>2 ± 3.9</td>
<td>94.99 ± 9.85</td>
<td>64.29 ± 6.94</td>
<td>-35.84 ± 5.74**</td>
</tr>
<tr>
<td>Barry-Albright Dystonia (BAD) Test Score</td>
<td>16.9 ± 1.48</td>
<td>14.64 ± 2.86</td>
<td>18.09 ± 3.02</td>
<td>4 ± 1.89</td>
<td>18.15 ± 1.65</td>
<td>20.89 ± 1.48</td>
<td>2 ± 0.43</td>
</tr>
<tr>
<td>PedsQL™ Physical Score</td>
<td>40.19 ± 3.77</td>
<td>36.55 ± 6.39</td>
<td>39.77 ± 7.60</td>
<td>3.22±6.18</td>
<td>42.19 ± 4.73</td>
<td>37.33 ± 5.62</td>
<td>-8.594 ± 6.36</td>
</tr>
<tr>
<td>PedsQL™ Psychosocial Score</td>
<td>70.89 ± 2.76</td>
<td>66.19 ± 3.05</td>
<td>71.97 ± 5.31</td>
<td>5.781 ± 3.61</td>
<td>73.47 ± 3.87</td>
<td>75.52 ± 4.31</td>
<td>-5.506 ± 7.68</td>
</tr>
<tr>
<td>PedsQL™ Total Score</td>
<td>60.13 ± 2.52</td>
<td>55.83 ± 3.29</td>
<td>60.68±4.57</td>
<td>4.851 ± 3.77</td>
<td>62.5 ± 3.40</td>
<td>61.44 ± 4.25</td>
<td>-7.203 ± 6.72</td>
</tr>
</tbody>
</table>

*All values are ± SE
**Significantly greater reduction than placebo treatment \( p<0.001 \)

**Iron Metabolism**

Eighteen months of DFP therapy normalized the expression levels of transferrin. Significantly reduced levels of transferrin at baseline (Figure 5-1A), \( p = 0.044 \), were elevated by 18 months of DFP therapy (Figure 5-1). No significant changes were seen in systemic iron levels after 18 months of DFP therapy. Serum iron levels, TIBC and transferrin saturation were largely unaffected by the treatment (Table 5-2). A small reduction was seen in serum ferritin levels, which remained within the reference range. The plasma levels of the alpha chain of hemoglobin remained significantly elevated
following 18 months of deferiprone treatment (Table 5-2), p = 0.003. sTfR levels remained slightly elevated following therapy (Table 5-2).

**Systemic Disease Burden**

Eighteen months of deferiprone therapy resulted in signs of a drop in acute inflammation and a rise in chronic inflammation. CRP levels which were significantly elevated at baseline compared to controls (Figure 5-2A), p = 0.01, were reduced after 18 months of therapy (Figure 5-2A). Significant increases in the inflammatory cytokines IL-10 (Figure 5-2C), p = 0.012 and TNFa (Figure 5-2D), p = 0.037, were seen following 18 months of treatment with DFP. Expression levels of IL-6 rose slightly, but not significantly, following 18 months of therapy. Additionally, 18 months of DFP therapy attenuated signs of systemic oxidative stress. The plasma levels of the antioxidant enzyme glutathione peroxidase that were significantly reduced at baseline (Figure 5-3A), p = 0.02, were elevated following therapy (Figure 5-3A). Conversely levels of the oxidative stress markers MDA and the free MDA pool which were increased in patient at baseline (Figure 5-3, B and C), p = 0.044, and p = 0.01, were reduced after 18 months of deferiprone treatment (Figure 5-3, B and C).

**Lipid Metabolism**

Notably low levels of apolipoproteins (Supplementary Table 0-10), were increased following 18 months of DFP therapy. Significant increases in the plasma levels of apolipoprotein Cl (Supplementary Figure 0-14A), p = 0.028, apolipoprotein ClII (Supplementary Figure 0-14B), p = 0.033, apolipoprotein ClIV (Supplementary Figure 0-14C) and apolipoprotein B100 (Supplementary Figure 0-14D), p = 0.012, were seen after therapy. Reduced levels of zinc alpha 2-glycoprotein (Figure 5-4D), p = 0.04, were elevated after 18 months of DFP therapy (Supplementary Table 0-10).

**Candidate Biomarkers**

A significant impact of 18 months of therapy was seen in a selection of the candidate biomarkers. The large increases observed in the plasma expression of the alpha, beta and gamma chains of fibrinogen at baseline (Figure 5-5A and Supplementary
Table 0-10), p<0.001, p<0.001 and p<0.001, were significantly reduced following therapy (Figure 5-10A, Supplementary Table 0-10), p< 0.001, p = 0.003 and p = 0.002. Reduced baseline plasma levels of transthyretin in PKAN patients (Figure 5-5B), p = 0.06, were significantly increased after 18 months of deferiprone therapy (Figure 5-10B), p = 0.012. Similarly, reduced levels of retinol-binding protein at baseline (Figure 5-4A), p = 0.02, were increased after treatment (Figure 5-10), p = 0.018.
Figure 5-10. Plasma expression of the proteins fibrinogen alpha chain (A), transthyretin (B) and retinol-binding protein (C) at baseline and following 18 months of deferiprone (DFP) therapy in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16 at baseline and n = 15 at 18 months) relative to controls (n = 15); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma fibrinogen alpha chain levels were significantly elevated in patients at baseline, p<0.001 (***, 2-tailed, unpaired, students t-test). These levels were significantly reduced following 18 months of deferiprone treatment, p<0.001 (***, 2-tailed, unpaired, students t-test). This elevation and subsequent reduction following therapy with deferiprone of fibrinogen alpha chain was also seen in a
patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN). Plasma retinol-binding protein levels were significantly reduced in patients at baseline $p = 0.018$ (*, 2-tailed, unpaired, students t-test). These levels were significantly elevated following 18 months of deferiprone treatment, $p = 0.012$ (*, 2-tailed, unpaired, students t-test). This reduction and subsequent elevation following therapy with deferiprone of retinol binding protein was also seen in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN). Plasma transthyretin levels were significantly reduced in patients, $p = 0.006$ (**, 2-tailed, unpaired, students t-test). These levels were significantly elevated following 18 months of deferiprone treatment, $p = 0.037$ (*, 2-tailed, unpaired, students t-test). This reduction and subsequent elevation following therapy with deferiprone of transthyretin was also seen in a patient with Phospholipase A2G6-Associated Neurodegeneration (PLAN).

5.5. Discussion

5.5.1. Assessment of the Systemic State of PKAN

This investigation builds on the work of Leoni et al. (2012) as well as previous studies in PD (Alberio et al., 2013; Andican et al., 2012; Chen et al., 2009; Chen et al., 2007; Saracchi et al., 2014; Wong et al., 2010), which highlight the potential for systemic disruptions accompanying neurodegeneration in NBIA disorders, by exploring a large panel of systemic biomarkers in a group of patients with PKAN. The findings of these investigation closely parallel the findings of a case study of a single patient with the late-onset parkinsonism-dystonia presentation of PLAN (Chapter 4).

Brain iron accumulation within the GP of PKAN patients did not have a significant impact on systemic iron levels. Serum iron, TIBC, hemoglobin concentration and transferrin saturation in PKAN patients all closely matched the control group. Evidence of changes to systemic iron trafficking were seen in the form of a significant decrease in Tf and a slight, but not significant increase in sTfR. As was the case in the patient with PLAN (Chapter 4), there were no signs of an increased systemic iron demand. Due to the lack of an obvious systemic source, the possibility exists that there is increased transferrin receptor (TfR) expression at the BBB due to an increased iron demand within the CNS. A portion of this TfR is released into the systemic circulation raising the circulating levels of sTfR. As transferrin responds to this increase it will be recycled at the BBB during receptor mediated endocytosis, lowering levels. This reduction in systemic transferrin levels is also a common finding in PD and was noted in the work of Logroscino et al. (1997) and
Tórsdóttir, Kristinsson, Sveinbjörnsdóttir, Snaedal, & Jóhannesson, (1999). Somewhat conflicting results have been reported for other serum iron parameters in PD. In the work of Tórsdóttir et al. (1999) and Madenci, Bilen, Arli, Saka, & Ak, (2012) no significant changes in serum iron, transferrin saturation or TIBC were found, but Logroscino et al. (1997) report that serum iron, serum ferritin, transferrin saturation and TIBC were reduced in the patients studied. A study by Hegde et al. (2004) provides a possible explanation for these contrary findings. They found that early in the disease course serum iron was not significantly altered, but a significant drop in serum iron was seen in severely affected PD patients. Drops in transferrin may be a more ubiquitous sign of disrupted iron trafficking in both PD and NBIA, but alterations in serum iron parameters may only occur later in the disease in age-onset patients with neurodegeneration, such as in PD. Further research needs to be conducted to determine if these alterations in transferrin levels are related to the accumulation of iron inside of the BG.

There was a systemic burden of neurodegeneration in the form of elevated oxidative stress and inflammation in the patients with PKAN. Though the levels of IL-6, TNFα and IL-10 were higher than what was seen in controls, they were not significantly elevated. However, a large elevation of CRP was seen in the patients, which is indicative of acute systemic inflammatory stress in PKAN. In addition to this inflammatory stress, elevations of the oxidative stress markers MDA and free MDA were observed. These elevations corresponded with significantly lower levels of glutathione peroxidase which were observed in patients. Similar reductions of glutathione peroxidase in the face of chronic oxidative stress were seen in individuals suffering from ongoing renal failure (Ceballos-Picot et al., 1996; Loughrey et al., 1994). This raises the possibility that chronic oxidative stress may lower the levels of endogenous antioxidant defenses in patients. Elevations in systemic oxidative stress and inflammation have also previously been reported in PD (Andican et al., 2012; Chen et al., 2009; Chen et al., 2007), which is further suggestive of a systemic burden in neurodegenerative disorders. Elevations in CRP correlated with increasing severity of motor symptoms. Suggesting a rising systemic disease burden over the course of disease progression.
Elevations in adiponectin and reductions in zinc alpha-2 glycoprotein (ZAG) and retinol binding-protein (RBP) as well as numerous apolipoproteins provide additional evidence for the possible alterations in systemic lipid metabolism noted in the PLAN patient (Chapter 4). Additionally, Leoni et al. (2012) revealed deficits in lipid metabolic pathways associated with a reduction in CoA in a study of patients with PKAN. This deficit was proposed to be due to impaired production of CoA due to PANK2 mutations. Collectively, alterations in the expression of these proteins provides further evidence for abnormalities in systemic lipid metabolism in PKAN, which could result in changes in the overall levels of HDL and LDL and their associated carrier lipoproteins. The alterations observed in the PKAN patients’ lipid metabolic proteins could be related to deficits noted by Leoni et al. (2012). The role of these proteins in lipid metabolism provides some insight into their potential for disruption in PKAN and other NBIA disorders. Increased adiponectin has been demonstrated to favour adipocyte formation as well as lipid deposition with in adipocytes (Turer & Scherer, 2012). Conversely elevated levels of ZAG have been proposed to downregulate the production of lipogenic enzymes and the upregulate lipolytic enzymes (Gong et al., 2009). In regard to its role in lipid metabolism, the dynamics of RBP have been reported to be the inverse of ZAG. It is elevated in patients with higher adiposity (Graham et al., 2006) and decreases fatty acid oxidation (Esteve, Ricart, & Fernández-Real, 2009). Unexpectedly, these two proteins, ZAG and RBP, which have inverse roles in regulating adiposity, are both lowered in patients. Additionally, this observed decrease is highly correlated between the two proteins suggesting a shared process influencing decreases in both proteins. It is unclear if this is due to changes in lipid metabolism in PKAN however.

One possible factor that may be influencing the observed differences in these proteins in patients with PKAN is the presence of a systemic inflammatory disease burden. In an inflammatory state, characterized by elevations in cytokines such as IL-6 and TNFα, the expression levels of both adiponectin and ZAG have also been reported to be reduced (Bao et al., 2005; Esteve et al., 2009). This phenomenon was not observed in our investigation or in our study of a patient with PLAN (Chapter 4). Adiponectin has also
been shown to be able to suppress inflammatory response from cells, such as macrophages (Turer & Scherer, 2012). This anti-inflammatory role is another possible explanation for its elevation. An elevation in adiponectin in the presence of chronic inflammation was also seen in the case study of the patient with PLAN (Chapter 4). Levels of RBP were demonstrated to be positively corelated with elevations in CRP and an inflammatory state (Jialal, Adams-Huet, Duong, & Smith, 2014). The reason for its reduction in patients in the presence of elevated levels of CRP is still needs to be explored in a disease state such as PKAN.

Another important factor to note is that ZAG is also secreted by the liver in the presence of thyroid hormone (T3) (Simó et al., 2014). RBP is also produced by the liver (Jialal et al., 2014) and is related to thyroid hormone through the shared carrier transthyretin (TTH). As the levels of thyroid hormone have not been measured in these patients it still needs to be confirmed if the reductions observed in ZAG and RBP are due to a reduction in thyroid hormone production in PKAN. Based on ALT levels altered liver health in the patients is an unlikely culprit. Another possible factor that has not been explored in NBIA is a reduction in retinol levels; as its name suggests RBP is a key retinol carrier. Visual abnormalities, including retinal degeneration and optic atrophy are a common symptom of NBIA in both PKAN and PLAN (Gregory et al., 2009; Hayflick et al., 2003; Hogarth, 2015; Levi & Finazzi, 2014). These symptoms could be influenced by low endogenous retinol levels, which is essential for maintaining proper visual function. Ultimately, the regulation of these proteins in a disease state such as PKAN still needs to be fully explored. As was the case in our assessment of a patient with PLAN (Chapter 4), the occurrence of multiple factors affecting these systems including chronic inflammation, dysfunction in proteins involved in lipid metabolism and weight loss and muscle wasting raise the possibility of a unique expression profile in patients which may be indicative both PLAN and PKAN.

Elevations in expression levels of fibrinogen and reductions in transthyretin (TTH) were observed in the patients with PKAN. Both of these proteins were highlighted by a recent large scale systemic review of biomarker studies in PD as potential systemic
biomarkers (Alberio et al., 2013). TTH has been linked to a number of neurological disorders including amyloidosis, Alzheimer’s disease and PD (Alberio et al., 2013; Velayudhan et al., 2012; Fleming, Nunes, & Sousa, 2009). TTH has been linked a diverse array of processes which includes both retinol and thyroid metabolism, which it directly acts in due to its role as a carrier protein (Fleming, Nunes, & Sousa, 2009; Han et al., 2011; Velayudhan et al., 2012). Additionally, it has been shown to potentially form protein aggregates (Fleming, Nunes, & Sousa, 2009). Some evidence of a possible protective role of elevated levels of TTH has been demonstrated in Alzheimer’s disease, where it could potentially limit amyloid beta plaque formation (Velayudhan et al., 2012). Low levels of TTH, which were observed in both the patient with PLAN (Chapter 4) as well as the cohort of patients with PKAN have been proposed as a disease marker (Han et al., 2011; Velayudhan et al., 2012). It was also observed to correlate inversely with disease severity in Alzheimer’s disease (Han et al., 2011; Velayudhan et al., 2012), which was observed in patients with PKAN as well as a patient with PLAN. Interestingly, work by Episkopou et al. (1993) in a mouse model with a genetic knockout at the TTH gene locus, ttr, demonstrated that a lack of TTH led to decreased levels of retinol, RBP and thyroid hormone. Low levels of RBP protein in conjunction with decreased levels of TTH were noted in the patients with PKAN. Furthermore, reduced ZAG expression in patients could be strongly influenced by a reduction in thyroid hormone levels. As noted above this raises the possibility that low levels of retinol and thyroid hormone are also present in PKAN. Symptoms of low retinol levels, such as optic atrophy and retinol degeneration are also frequently observed in PKAN (Gregory et al., 2009; Hayflick et al., 2003; Hogarth, 2015; Levi & Finazzi, 2014). In addition to these findings in PKAN, a large reduction in TTH levels as well as a slight reduction in RBP protein levels were seen in the case study of PLAN (Chapter 4). Based on these findings, strong consideration should be give to the exploration of both thyroid hormone and retinol levels in NBIA patients as possible sources of systemic disruption.

The final marker of interest noted in our study was fibrinogen. All three chains were significantly elevated in patients with PKAN and the patient with PLAN (Chapter 4). Limited information is available as to a possible reason for elevations in fibrinogen in
In Alzheimer’s disease it has been reported to pass through the BBB and as proposed to be causing damage via fibrin deposition (Ryu & McLarnon, 2009), whether this also occurs in NBIA is unclear. Interestingly, elevations in fibrinogen, have also been reported to be associated with an increased risk of developing PD (Wong et al., 2010). Further research needs to be performed to confirm the exact role of these proteins in NBIA, but the fact that alterations in adiponectin, RBP, TTH and fibrinogen were also observed in both PKAN and PLAN is suggestive that disruptions in the expression of these proteins may be a shared hallmark for NBIA disorders.

Significant correlations were observed between the severity of the disease state in patients and disrupted systemic levels of fibrinogen, transthyretin, ZAG and RBP. Elevations in all three fibrinogen chains correlated to both iron loading in the GP and the severity of motor symptoms. A similar correlation was seen between low levels of TTH and the severity of motor symptoms. These correlations provide further support or both fibrinogen and TTH as candidate systemic biomarkers in NBIA. However, one major concern with there use is the fact that the expression range of these proteins in patients overlaps with the control expression range. An assessment of these overlapping expression ranges was done using a 3-factor visual analysis. Patients and controls were visually stratified based on expression levels of fibrinogen, TTH and disease severity. Based on this 3-factor stratification a severely impacted patient population is clearly seen with low transthyretin and high fibrinogen levels. As disease severity becomes more pronounced the respective elevation and reduction of fibrinogen and transthyretin is more apparent. Though the expression range in the PKAN patients overlaps with the control range for these proteins, severely affected patients express both of these proteins at levels outside of the control range. This demonstrates the feasibility of these proteins as disease markers despite the overlap.

A significant relationship was also seen between disease severity and RBP as well as ZAG. Increases in ZAG correlated with an increase in iron loading in the GP and increased severity of motor symptoms. Increases in RBP also correlated with increasing iron loading in the BG in patients. This relationship represents an interesting dynamic in
patients, levels of these proteins which were significantly lower than the control group rise towards control levels as the disease progresses. This relationship makes these markers poor choices for analysis in severely affected patients. However, significant decreases in both proteins in less severely affected patients make them promising candidates for screening in the early stages of disease. The role of ZAG in controlling adiposity, in particular lipolysis means that elevations in this protein in more severe cases may also be representative marker of wasting and weight loss in patients. Evidence for this hypothesis was the observation of significant elevations in ZAG in a case study of a progressed patient with PLAN (Chapter 4). Finally, significant intermarker correlations were seen between the disruptions in fibrinogen, TTH, ZAG and RBP. This is suggestive of a unique expression profile in regard to these proteins in patients. As noted above, ZAG and RPB are significantly lower in less severely affected patients and fibrinogen is higher and TTH is lower in more severely affected patients. The collective expression profile of these 4 proteins may be able to distinguish patients based on disease severity.

5.5.2. Efficacy of Deferiprone Therapy

Based on the markers assessed in this substudy, DFP was well tolerated by patients during the extended 18-month trial. This was also the case in previous trials of DFP in NBIA and PD (Abbruzzese et al., 2011; Devos et al., 2014; Fasano et al., 2012; Forni et al., 2008; Zorzi et al., 2011). DFP treatment was highly successful in lowering brain iron accumulation in the GP based on R2* MRI assessment. A large drop in GP brain iron levels were seen in treated, but not in placebo patients. All of the treated patients that received a follow-up MRI showed a reduction in brain iron accumulation in the GP. This builds on previous findings of efficacy of DFP in reducing brain iron accumulation after both 6 months and 1 year of therapy and demonstrates that 18 months of therapy is highly efficacious in lowering brain iron accumulation in NBIA. Based on changes in BAD score over the course of 18 months, patients treated with DFP showed a slower disease progression, with lower increases in the symptoms of dystonia during the trial than placebo patients. However, due to the variability in BAD score changes in the patients this difference was not significant. Of the 20 patients treated with DFP, 2 patients saw a slight
decrease in the symptoms of dystonia, 4 showed no change in symptoms and 16 patients had a minor increase. The dynamic originally presented by Devos et al. (2014) of an increased efficacy of DFP based on early intervention still needs to be explored in this study population. This is an interesting dynamic because during early intervention it is more likely that sufficient functionality remains within the BG to allow for some recovery of motor function. It is likely that as the disease progresses fewer functional neurons would remain to enable this recovery. Stratification of patients based on disease severity at the initiation of DFP therapy to determine if early intervention improved efficacy is an ongoing area of research. No significant changes were noted in response to DFP therapy based on the PedsQL™ assessment. One limitation to the use of PedsQL™ in this study is the high number of patients that were unable to complete the survey themselves. Parental assessment of the patient’s condition may have limited the sensitivity of the survey.

Eighteen months of DFP treatment did not significantly change serum iron levels, TIBC or transferrin saturation in the patients. A slight, but not significant decrease in serum ferritin was noted. Serum ferritin levels in patients were in the lower end of the reference range prior to the initiation of therapy and this decrease did not result in a drop outside of the reference range. A similar drop in serum ferritin was observed by Devos et al. (2014) during DFP treatment of a group of patients with PD. This drop was attributed to a redistribution of hepatic and other iron stores due to DFP mobilization (Devos et al., 2014). Contrary to what was seen in the patient with PLAN (Chapter 4), DFP treatment significantly raised transferrin levels in PKAN patients. The presence of DFP and a reduction in brain iron may have normalized iron trafficking in treated patients, but the exact relationship between these factors needs further investigation. Overall, the therapy was well tolerated based on systemic iron trafficking markers with minimal impact on systemic iron levels.

Some mitigation to the system disease burden was provided by DFP therapy. CRP, MDA and free MDA were all lowered by therapy to the point where they were no longer significantly elevated over the control population. A rise was seen in the proinflammatory
cytokines IL-6 and TNFα. This was accompanied by a rise in IL-10, which has previously been demonstrated as an immune response to limit chronic inflammation (Mizoguchi, Mizoguchi, Takedatsu, Blumberg, & Bhan, 2002), which may be occurring these patients. As was noted in the study of a patient with PLAN (Chapter 4) this increase over time of inflammatory cytokines may be characteristic of the disease progression in patients and not of DFP therapy. In addition to the findings in a case study to PLAN, elevated systemic inflammatory markers have also been noted in other neurodegenerative diseases (Andican et al., 2012; Chen et al., 2009; Chen et al., 2007), which is suggestive of a link between chronic inflammation and the progression of neurodegenerative symptoms.

DFP therapy also reduced abnormalities seen in a number of the proteomic markers assessed in this study. This included reductions in fibrinogen and adiponectin as well as elevations in TTH, ZAG and RBP. Further delineation for the underlying causes of abnormalities in these proteins in PKAN needs to be done before the impact of these changes during DFP on the disease state in the PKAN can be determined. Interestingly, many of the candidate proteomic marker levels moved towards the control population levels during DFP therapy. Fibrinogen was significantly decreased and TTH was significantly increased in patients who had been treated with 18 months of DFP. The reduction in fibrinogen mirrored the previously demonstrated relationship between fibrinogen and GP iron accumulation. A reduction in both factors was seen in treated patients. This reduction was also seen in a patient with PLAN following DFP therapy, providing further evidence of a direct relationship between DFP treatment and this reduction. However, until the underlying reason for fibrinogen elevations in NBIA is determined it is unclear how DFP may have influenced Fibrinogen levels. This is also the case for increased transthyretin levels.

Based on the positive correlations observed between the severity of motor symptoms and the plasma expression levels of ZAG as well as RBP it is possible that elevations in these two proteins are due to continued disease progression in the patients. Disease progression may have been slightly delayed by DFP treatment, but it continued over the course of the trial based on the BAD score measurements in the patients. Based
on their previously demonstrated correlations with disease severity, it is possible that these elevations in ZAG and RBP accompanied increasing disease severity in the patients. Another possibility is that previously alterations in lipid dynamics due to PANK2 mutations or due to weight loss and muscle wasting in patients are being influenced by therapy. This is supported by observed elevations in previously low levels of apolipoproteins towards control levels and a matching reduction in adiponectin towards the control patient range. As was the case for both fibrinogen and TTH, further research needs to be done to determine the reason for changes in the expression of systemic lipid metabolic proteins before the impact of DFP therapy on their levels can be fully understood.

5.6. Conclusions

This investigation confirmed and expanded the findings of a previous case study of a single patient with PLAN (Chapter 4) in a large cohort of patients with PKAN. It was robustly shown that brain iron accumulation NBIA has minimal impact on systemic serum iron levels, TIBC and transferrin saturation. This was accompanied by evidence of alterations in systemic iron trafficking in the form of reductions in transferrin and a slight elevation in sTfR, which occurred in the absence of signs of an abnormal iron demand systemically. Further evidence was found for a systemic burden of neurodegeneration in NBIA. Both elevations in oxidative stress and inflammatory markers were seen in the patients with PKAN. Alterations in the expression levels of apolipoproteins as well as adipocytokines reinforced the possibility of disruptions in systemic lipid metabolism in PKAN. A small group of markers were revealed by proteomic analysis to be strong candidate blood-based biomarkers. Notably, both fibrinogen and TTH correlated with disease severity in patients and in severely affected patients were expressed at levels outside of the control range. Additionally, reductions in ZAG and RBP may be present early in the disease progression in less severely affected patients. Evidence was highlighted that reductions in TTH may be responsible for low levels of ZAG and RBP in patients. Reduced levels of RBP and TTH also raises the possibility of lower systemic levels of retinol and thyroid hormone. Further screening should be performed in PKAN patients and other patients with NBIA to determine if systemic deficiencies in either of these factors exists.
DFP was well tolerated in patients and was shown to be highly efficacious in lowering brain iron accumulation in the GP following 18 months of treatment. It had minimal impact on systemic iron levels in patients and normalized the expression levels of transferrin. Additionally, it provided some reduction in the systemic disease burden by lowering CRP, MDA and free MDA in patients. A rise in inflammatory cytokines was seen after 18 months, but this may have been relating to ongoing disease progression in patients as a similar rise was demonstrated in a patient with PLAN in the absence of DFP. DFP also impacted the expression levels of the candidate biomarkers noted above, but further investigation is needed to understand how they are related to the disease state in NBIA to determine the relationship of DFP therapy to these changes. Due to its high efficacy in removing brain iron accumulation as well as its potential to mitigate the systemic disease burden, DFP is a promising therapeutic tool in the treatment of NBIA.
References


Deepayan Sarkar. (2008). Lattice: Multivariate Data Visualization with R. New York:
Springer.


# Appendix

## Appendix A. Summary of Characterized NBIA Disorders

### Supplementary Table 0-1. Overview of Characterized NBIA Disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Gene</th>
<th>Protein* (Provided by Hugo Gene Nomenclature Committee (HGNC))</th>
<th>Iron Related Protein</th>
<th>Relative Prevalence (Remaining cases are idiopathic NBIA)</th>
<th>Inheritance</th>
<th>Sites of Iron Accumulation (Levi &amp; Finazzi, 2014)</th>
<th>Onset and Progression (Gregory &amp; Hayflick, 2013)</th>
<th>Progression and Presentation (Gregory &amp; Hayflick, 2013; Levi &amp; Finazzi, 2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pantocchinate Kinase Associated Neurodegeneration (PKAN)</td>
<td>PANK2 (Zhou et al., 2001) (NCBI Gene ID: 80025)</td>
<td>pantocchinate kinase 2</td>
<td>N</td>
<td>50%</td>
<td>Autosomal</td>
<td>globus pallidus</td>
<td>Early childhood onset, rapid progression</td>
<td>Early gait abnormalities, dystonia, dysthria, spasticity and retinal degeneration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atypical PKAN: Delayed onset and slower progression</td>
<td></td>
</tr>
<tr>
<td>Phospholipase A2G6-Associated Neurodegeneration (PLAN)</td>
<td>PLA2G6 (Morgan et al., 2006) (NCBI Gene ID: 8398)</td>
<td>phospholipase A2 group VI (PLA2B)</td>
<td>N</td>
<td>20%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra*</td>
<td>Infantile Neuroaxonal Dystrophy (INAD) Very early childhood onset and rapid progression</td>
<td>Hypotonia, visual disturbance, motor and mental retardation, spasticity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atypical NAD and PARK1A: Delayed onset and slower progression</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial membrane protein-associated neurodegeneration (MPAN)</td>
<td>C9orf12 (Hartig et al., 2011) (NCBI Gene ID: 83636)</td>
<td>chromosome 19 open reading frame 12</td>
<td>N</td>
<td>10%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra</td>
<td>Childhood to early adulthood onset with slow progression</td>
<td>Dysthria, spasticity gait abnormalities, dystonia, and parkinsonism with some cognitive decline</td>
</tr>
<tr>
<td>Beta-propeller protein-associated neurodegeneration (BPAN)</td>
<td>WDR45 (Hartig et al., 2011) (NCBI Gene ID: 11152)</td>
<td>WD repeat domain 45 (Beta-propeller protein)</td>
<td>N</td>
<td>7%</td>
<td>X-linked dominant</td>
<td>globus pallidus and substantia nigra</td>
<td>Early onset developmental delay in childhood with rapid progression of parkinsonism in adolescence or adulthood</td>
<td>Parkinsonism, dystonia, dementia,</td>
</tr>
<tr>
<td>Fatty acid hydroxylase-associated neurodegeneration (FAHN)</td>
<td>FAN2 (Michael C. Kruer et al., 2010) (NCBI Gene ID: 79152)</td>
<td>fatty acid 2-hydroxylase</td>
<td>N</td>
<td>1%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra**</td>
<td>Childhood onset with slow progression</td>
<td>Gait abnormalities, dystonia, and parkinsonism, optic atrophy and some cognitive decline</td>
</tr>
<tr>
<td>Coenzyme A synthase associated neurodegeneration (CoPAN)</td>
<td>COASY (Busi et al., 2014) (NCBI Gene ID: 83437)</td>
<td>Coenzyme A synthase</td>
<td>N</td>
<td>Rare &lt; 1%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra</td>
<td>Early onset with slow progression</td>
<td>Oro-mandibular dystonia with dysthria and parkinsonism, cognitive impairment, axonal neuropathy</td>
</tr>
<tr>
<td>Kufor-Rakeb syndrome</td>
<td>ATP13A2 (Schneider et al., 2010) (NCBI Gene ID: 23400)</td>
<td>ATPase 13A2</td>
<td>N</td>
<td>Rare &lt; 1%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra***</td>
<td>Juvenile onset (small case number limits information on disease progression)</td>
<td>Dystonia dementia, gait palsy and myoclonus and parkinsonism</td>
</tr>
<tr>
<td>Woodhouse-Sakati syndrome</td>
<td>DCAF17 (Alazami et al., 2008) (Formerly C2orf57) (NCBI Gene ID: 80067)</td>
<td>DDB1 and CUL4 associated factor 17</td>
<td>N</td>
<td>Rare &lt; 1%</td>
<td>Autosomal</td>
<td>globus pallidus and substantia nigra***</td>
<td>Limited information available</td>
<td>Dystonia, dysthria and cognitive decline and endocrine disruption including diabetes mellitus and hypogonadism and alopecia</td>
</tr>
<tr>
<td>Neuroferritinopathy</td>
<td>FTL (Curtis et al., 2001) (NCBI Gene ID: 2512)</td>
<td>Ferritin Light Chain</td>
<td>Y</td>
<td>Rare &lt; 1%</td>
<td>Autosomal</td>
<td>Dense ferritin-Fe spheroid inclusions in dentate nuclei, globus pallidus, putamen, caudate, thalamus, and red nuclei</td>
<td>Adult onset and slower progression</td>
<td>Chorea or dystonia, dysthria in some cases cognitive decline</td>
</tr>
<tr>
<td>Aceruloplasminemia</td>
<td>CP (Harris et al., 1996) (NCBI Gene ID: 1356)</td>
<td>Ceruloplasmin</td>
<td>Y</td>
<td>Rare &lt; 1%</td>
<td>Autosomal</td>
<td>Dense ferritin-Fe spheroid inclusions in dentate nuclei, globus pallidus, putamen, caudate, thalamus, and red nuclei</td>
<td>Adult onset and slower progression</td>
<td>Diabetes, retinal degeneration anemia, dystonia, tremors, chorea and ataxia dysthria</td>
</tr>
</tbody>
</table>

*In a selection of patients
**in some patients
***in few patients
Appendix B: Fluorescent Imaging Setup

The initial fluorescent imaging setup utilized in these mice involved the dual staining of brain slices. The first stain utilized was an anti-tyrosine hydroxylase antibody targeted using Cy3 to locate the SN and assess dopaminergic activity. This was accompanied by a second stain of either anti-ubiquitin or anti-alpha synuclein antibodies targeted using Alexa488. As the spectrums of Alexa488 and YFP overlap nearly 100%, the presence of endogenous YFP fluorescence in the mice was not noted until a portion of the imaging in the mice was already completed. Upon confirmation of the presence of THY1-YFP in the mice a set of unstained images were taken. The fluorescent intensity of these images was subtracted from the previously taken stained images. As no consistent difference could be found between the two image sets, the presence of ubiquitin or alpha synuclein could not be confirmed and as such are not commented on in this work. Further research efforts will need to be done to verify the initial imaging and confirm if alpha-synuclein and ubiquitin are present in the observed spheroids.

Appendix C. Genotyping PCR Details and Reference Gel

Supplementary Table 0-2. PCR master mix utilized for genotyping of B6.C3-Pla2g6\textsuperscript{m1J/CxRwb} Mice

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Go Taq™ 2x Master Mix</td>
<td>12.5</td>
</tr>
<tr>
<td>Primer Mix (33 uM of each primer)</td>
<td>0.75</td>
</tr>
<tr>
<td>Digested Ear Punch DNA</td>
<td>1.0</td>
</tr>
<tr>
<td>ddH2O</td>
<td>10.75</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>

Supplementary Table 0-3. PCR cycle settings used for genotyping of B6.C3-Pla2g6\textsuperscript{m1J/CxRwb} Mice

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>Amplification (34 Cycles)</td>
<td>Denaturation 94 °C, Annealing 57 °C, Extension 72 °C</td>
<td>30 Seconds, 1 minute, 1 minute and 30 seconds</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>Holding</td>
<td>4 °C</td>
<td>-N/A</td>
</tr>
</tbody>
</table>
Supplementary Figure 0-1. Reference genotyping gel used for B6.C3-Pla2g6<sup>m1J/CxRwb</sup> Mice. Homozygous mice positive for the IAP insertion in the PLA2G6 gene present with a single band at ~150 bp. Wildtype mice present with a single band at ~280bp. Heterozygous mice present with 2 bands.
Appendix D. Open Field Test Setup

Open Field Test Overview

I. Open Field Test Setup

A. Open Field Apparatus

- Open field test apparatus under red light (A). Mice underwent the open field test at 30, 60, 80 and 90 days of age.
- Mice are placed inside of the open field test arena for the purpose of recording general locomotion and exploratory behaviour. Each test is videotaped for later analysis.
- Each mouse is placed in isolation (B) for an initial 3 minutes to acclimatize to the new environment and to avoid letting stress from handling influence results.
- Following the isolation period each mouse is recorded for 5 minutes as it explores and moves around the cage (C).

B. Isolation Portion of Test

C. Exploration Portion of Test

II. Open Field Test Analysis

- All videos analyses were performed blinded.
- The mouse’s position in each slide of the video was used to determine its velocity and distance travelled during the test.
- An additional analysis of where the mouse spend its time during the test was performed. The cage was separated into an interior and exterior portion of equal areas and the time the mouse spent in each portion was noted (D).
- Data was pooled by age and genotype.

D. Interior vs. Exterior Division of Test Chamber

Exterior Area

Interior Area

Supplementary Figure 0-2. An overview of the setup and utilization of the open field locomotor test.
Appendix E. Wire Hang Test Setup

Wire Hang Test Overview

I. Wire Hang Test Setup

- Constructed a wire hang apparatus (A)
- At 60, 80 and 90 Days of old. Mice hang suspended 40 cm above a ~1 inch thick layer of bedding.
- Mice are placed on the wire for a 3 minute (180s long test). Every time the mouse left the wire it was given a 5 second rest and then returned to the wire.
- To avoid generating an artificially short time on wire at end of test, mice were giving up to 1 minute of additional time if they remained on the wire at the end of the test.
- Behaviour on the wire was videotaped for each test.

A. Wire Hang Apparatus

II. Wire Hang Test Analysis

- Blinded analysis of each test was performed.
- All notable behaviour on the wire (B and C) was record and the time at which it occurred during the test was noted.
- Data was pooled by age and genotype.

B. Controlled Behaviour Noted During Wire Hang Test

- Balance
- Drop
- Reach

C. Uncontrolled Behaviour Noted During Wire Hang Test

Supplementary Figure 0-3. An overview of the setup and utilization of the wire hang test of motor strength and coordination.
Appendix F. Fluorescent Imaging Location Reference Guide

Fluorescent Imaging Reference Locations

- 5 Images were taken of each side of coronal sections of the SN.
- Two were taken at 10 x magnification of the VTA (A) and the SN (B).
- Three were taken at 20 x magnification of the medial portion of the SN (C), the central portion of the SN (D) and the lateral portion of the SN (E).
- Analysis was performed using the images taken at 20 x magnification. Area selection during analysis ensured that no overlap between counted areas occurred. VTA neurons were omitted from the analysis by area selection.
- The medial and lateral portion of the SNr was captured in (D) and (E) respectively.

Supplementary Figure 0-4. An overview of the imaging locations used during fluorescent microscopy.
Appendix G Additional Mouse Graphs

Supplementary Figure 0-5. Average mouse weights over time for wildtype (PLA2<sup>+/+</sup>, n = 11 (9F/2M)), heterozygous (PLA2<sup>+</sup>/<sup>-</sup>, n= 11(5F/6M)) and homozygous (PLA2<sup>-/-</sup>, n = 16(8F/8M)) mice. Weights were grouped by genotype (A) or separated into female (B) and male (C) mice. Error bars represent SE.
Supplementary Figure 0-6. Mouse wire hang test results for wildtype (PLA2R+/+, n = 6 (5F/1M)), heterozygous (PLA2R+/-, n= 5 (3F/2M)) and homozygous (PLA2R−/−, n = 6(3F/3M)) mice. At 60, 80 and 90 days of age mice were placed on a suspended wire for 3 min and test behaviour was recorded. Homozygous mice display reduced lateral movement on the wire (A); as represented by the number of times they reached the end of the test wire, during their test at all ages as compared to wildtype and heterozygous mice. By 90 days of ages homozygous mice had a significantly increased number of falls (B) during their tests compared to wildtype, $p = 0.001$, **, and heterozygous mice, $p = 0.007$, ** (Anova with Tukey’s HSD test). The combined Falls/Reaches score of the homozygous mice was significantly reduced compared to wildtype, $p = 0.004$, **, and heterozygous mice, $p = 0.04$, * (Anova with Tukey’s HSD test). At 90 days of age none of the homozygous mice were able to pull themselves up and balance on the wire, while all of the wildtype and heterozygous mice could (wildtype, $p< 0.001$, ***, and heterozygous mice, $p = 0.006$, *** (Anova with Tukey’s HSD test)). Error bars represent SE.
Supplementary Figure 0-7. Additional mouse wire hang test results for wildtype (PLA2^{+/+}, n = 6 (5F/1M)), heterozygous (PLA2^{+/−}, n= 5 (3F/2M)) and homozygous (PLA2^{−/−}, n = 6(3F/3M)) mice. At 60, 80 and 90 days of age mice were placed on a suspended wire for 3 min and test behaviour was recorded. At 90 days of age the strength of the homozygous mice was reduced as measured by their weight adjusted time on the wire (impulse) during the test. Their total impulse was significantly reduced compared to wildtype \( p = 0.02 \), *, and heterozygous mice at 90 days, \( p = 0.05 \), * (Anova with Tukey’s HSD test). The maximum impulse without falling off of the wire was significantly reduced in the homozygous mice compared to wildtype \( p = 0.036 \), *, and heterozygous mice at 90 days, \( p = 0.046 \), * (Anova with Tukey’s HSD test). Error bars represent SE.
Supplementary Figure 0-8. Mouse open field test results for wildtype (PLA2^+/+, n = 5 (4F/1M)), heterozygous (PLA2^-/-, n = 5 (3F/2M)) and homozygous (PLA2^-/-, n = 6(3F/3M)) mice. Mice were allowed to freely explore an open field test arena (42 x 20 cm) for 5 min under red light. No notable differences were seen by genotype in the total distance moved during the test (A), the average velocity of the mouse during the test (B), the percent of the test spent moving (C) or the positioning of the mouse during the test (D). Repeated testing increased the portion of the test that mouse felt comfortable to be in the interior of the test arena. Error bars represent SE.
Supplementary Figure 0-9. Additional Representative fluorescent images of the substantia nigra in mice visualized using Cy3 targeted to anti-tyrosine hydroxylase antibodies in 60-day old homozygous (PLA2/−) mice and 90-day old homozygous (PLA2/−). Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing using previously captured Cy3 and SN autofluorescent spectrums. Homozygous mice at 90 days of age (D-F) show a marked reduction in the number of TH positive neurons inside of the lateral portion of the SN pars Reticulate (SNr) in comparison to homozygous mice at 60 days of age (A-C).
Supplementary Figure 0-10. Tyrosine hydroxylase (TH) positive neuron counts of Cy3 targeted to anti-TH antibodies in the substantia nigra (SN) of 60-day old homozygous (PLA2^+/^+, n = 30 slides from 3 mice (2F/1M)) and 90-day old homozygous mice (PLA2^-/-, n = 50 slides of SNc from 6 mice (3F/3M)). Images were taken on a Nikon Eclipse Ti-E inverted microscope attached to a C1si spectral confocal system using a Nikon 20x CFI PLAN Apochromat objective. Images were processed using linear unmixing with previously captured Cy3 and SN autofluorescent spectrums and a count was taken in each region using ImageJ. A reduction in TH positive neurons was seen in the lateral portion of the SN pars reticulata (SNr) in 90-day old homozygous mice compared to 60-day old homozygous mice (A), p<0.001, ***, (2-tailed, unpaired, student’s t-test). This reduction was concentrated in a single population of TH positive neurons seen at Bregma locations < -3.2 (C). Error bars represent SE.
Supplementary Figure 0-11 Correlations between the mean intensity of Cy3 targeted to anti-tyrosine hydroxylase antibodies in the substantia nigra pars reticulata (SNr) and the final Falls/Reaches score on the wire hang test of 90-day old wildtype mice (PLA2^{++}, n = 5 (4F/1M)), heterozygous mice (PLA2^{+/-}, n = 4 mice (3F/1M)), and homozygous mice (PLA2^{-/-}, n = 6 mice (3F/3M)). A significant correlation was seen between the test score and the mean intensity in the substantia nigra pars reticulata (SNr) medial, $p = 0.03$, SNr lateral, $p = 0.04$, and the full SNr, $p = 0.02$, (Pearson’s Correlation).

Appendix H. Removed Control Details

A single control patient had a previous history of anorexia nervosa, but they were currently in the weight recovery period of the disease and was back at a healthy weight at the time of enrollment. Preliminary biomarker analysis revealed massively elevated cytokine levels in the patient. This inflammation is implicated in the pathogenesis of anorexia nervosa (Solmi et al., 2015). Due to the ongoing presentation of elevated inflammatory markers, this patient was omitted from the control group.
Appendix I. Deferiprone Trial Monitoring Details for a Patient with PLAN

Supplementary Table 0-4. Blood markers monitored over the course of deferiprone therapy in a patient with PLA2G6 associated neurodegeneration.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Average</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Blood Cells</td>
<td>7.3</td>
<td>4.0-10.0 (giga/L)</td>
</tr>
<tr>
<td>Red Blood Cells</td>
<td>5.4</td>
<td>4.20-5.40 (tera/L)</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>162.0</td>
<td>133-165 (g/L)</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.5</td>
<td>0.38-0.50</td>
</tr>
<tr>
<td>Mean Cell Volume</td>
<td>88.0</td>
<td>82-98 (fl)</td>
</tr>
<tr>
<td>Mean Corpuscular Hemoglobin</td>
<td>29.8</td>
<td>27.5-33.5 (pg)</td>
</tr>
<tr>
<td>Red Blood Cell Distribution</td>
<td>13.2</td>
<td>11.5-14.5 (%)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>5.4</td>
<td>2.0-7.5 (giga/L)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.3</td>
<td>1.0-4.0 (giga/L)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.5</td>
<td>0.1-0.8 (giga/L)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.1</td>
<td>0.0-0.7 (giga/L)</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.0</td>
<td>0.0-0.2 (giga/L)</td>
</tr>
<tr>
<td>Platelet Count</td>
<td>245.0</td>
<td>150-400 (giga/L)</td>
</tr>
<tr>
<td>Serum Ferritin</td>
<td>68.0</td>
<td>15-300 (ug/L)</td>
</tr>
<tr>
<td>Alanine Aminotransferase</td>
<td>&lt;10</td>
<td>&lt;50 (U/L)</td>
</tr>
<tr>
<td>Serum Iron</td>
<td>10.0</td>
<td>10-33 (umol/L)</td>
</tr>
<tr>
<td>Total Iron Binding Capacity</td>
<td>57.0</td>
<td>37-72 (umol/L)</td>
</tr>
<tr>
<td>Transferrin Saturation</td>
<td>0.2</td>
<td>0.2-0.55</td>
</tr>
<tr>
<td>Cortisol</td>
<td>N/A</td>
<td>138 – 690 (nmol/L)</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>N/A</td>
<td>0-2 (mg/L)</td>
</tr>
<tr>
<td>Lactate Dehydrogenase</td>
<td>N/A</td>
<td>105 – 333 (IU/L)</td>
</tr>
</tbody>
</table>

Appendix J. ELISA Standard Curves

Supplementary Table 0-5. TNFa, IL-10 and IL-6 Standard Curve Setups

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Standard Curve Range (pg/mL)</th>
<th>Number of Points in Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>TNFα</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>18.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Appendix K. Serum Iron and TIBC Adjusted Volumes

Supplementary Table 0-6. Adjusted serum iron and TIBC volumes for use in a 96-well plate.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Percent of Final Solution</th>
<th>Volume in 96-Well Plate (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid (1.3 mol/L)</td>
<td>3.7</td>
<td>11.25</td>
</tr>
<tr>
<td>Acetate Buffer in DMSO (0.087 mol/L, pH 4.65)</td>
<td>74.1</td>
<td>225</td>
</tr>
<tr>
<td>Serum Sample</td>
<td>18.5</td>
<td>56.25</td>
</tr>
<tr>
<td>Chromogen (Ferene)</td>
<td>3.7</td>
<td>11.25</td>
</tr>
</tbody>
</table>
Supplementary Table 0-7. Overview of plasma proteins successfully measured using multiple reaction monitoring mass spectrometry proteomics

<table>
<thead>
<tr>
<th>Protein</th>
<th>Analysis Method</th>
<th>Category</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Secreted by adipose tissue for the regulation of glucose levels and lipid metabolism</td>
</tr>
<tr>
<td>Alpha1 Antitrypsin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Involved in the regulation of trypsin, chymotrypsin and plasminogen</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Regulation of blood coagulation</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein AI</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein AIV</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein B100</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein CI</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein CII</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein CIII</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein CIV</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein D</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein F</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein L1</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein M</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Apolipoprotein a</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Various roles in lipid, fatty acid and cholesterol transport and metabolism</td>
</tr>
<tr>
<td>Beta-2-microglobulin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>MHC component. Diverse range of other proposed functions.</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Iron Metabolism and Transport</td>
<td>Copper binding and ferroxidase activity.</td>
</tr>
<tr>
<td>Cholinesterase</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Esterase responsible for the inactivation of acetylcholine</td>
</tr>
<tr>
<td>Protein</td>
<td>Analysis Method</td>
<td>Category</td>
<td>Function</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Fibrinogen Alpha Chain</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Other</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen Beta Chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibrinogen Gamma Chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione Peroxidase 3</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Antioxidant</td>
<td>Catalyzes the reduction of peroxides using glutathione.</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Iron Metabolism and Transport</td>
<td>Binding and transport of free hemoglobin in circulation</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemopexin</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Iron Metabolism and Transport</td>
<td>Binding and transport of free heme in circulation</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropilin 2</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Other</td>
<td>Receptor involved in the activity of various endothelial growth factors</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipid Transfer Protein</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Lipid Metabolism</td>
<td>Involved in the transport of lipid molecules</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retinol-binding Protein 4</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Other</td>
<td>Binds retinol and mediates its transport throughout the body</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serotransferrin</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Iron Metabolism and Transport</td>
<td>Primary protein responsible for binding and transport of ferric iron in circulation</td>
</tr>
<tr>
<td></td>
<td>MRM-Proteomics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>UVic Genome BC Proteomic Center</td>
<td>Other</td>
<td>Regulation of osmotic pressure of blood. Transport of plasma Cations and other molecules.</td>
</tr>
<tr>
<td>Protein</td>
<td>Analysis Method</td>
<td>Category</td>
<td>Function</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------------------------------</td>
<td>-------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Serum Amyloid A-1.and A.-2 Proteins</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Acute phase protein. Interacts with apolipoproteins to trigger amyloidosis</td>
</tr>
<tr>
<td>Serum Amyloid A-4 Protein</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Acute phase protein. Interacts with apolipoproteins to trigger amyloidosis</td>
</tr>
<tr>
<td>Serum Amyloid P-component</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Acute phase protein. Interacts with apolipoproteins to trigger amyloidosis</td>
</tr>
<tr>
<td>Sex Hormone-binding Globulin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Binds and transports various sex hormones.</td>
</tr>
<tr>
<td>Transthyretin</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Transport protein of thyroxine and retinol-binding protein.</td>
</tr>
<tr>
<td>Zinc Alpha 2 Glycoprotein</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Lipid Metabolism</td>
<td>Involved in the lipid breakdown in adipocytes.</td>
</tr>
<tr>
<td>Vitamin-D-binding Protein</td>
<td>UVic Genome BC Proteomic Center MRM-Proteomics</td>
<td>Other</td>
<td>Vitamin D transport and storage.</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>SISCAPA Immunoassay Enhanced MRM Proteomics</td>
<td>Iron Metabolism and Transport</td>
<td>Oxygen transport molecule. Found as a tetramer inside of erythrocytes.</td>
</tr>
<tr>
<td>C-Reactive Protein</td>
<td>SISCAPA Immunoassay Enhanced MRM Proteomics</td>
<td>Inflammation</td>
<td>Released from the liver in response to inflammation.</td>
</tr>
<tr>
<td>Soluble Transferrin Receptor</td>
<td>SISCAPA Immunoassay Enhanced MRM Proteomics</td>
<td>Iron Metabolism and Transport</td>
<td>Receptor responsible the internalization and uptake of transferrin. Soluble levels are proposed to be a marker of iron metabolism and are used as an indicator of anemia.</td>
</tr>
</tbody>
</table>
Appendix M Additional MRM Information

MRM Overview

Multiplex multiple reaction monitoring (MRM) proteomics involves the application of selected reaction monitoring (SRM) proteomics to multiple target ions. SRM is a tandem mass spectrometry technique that involves the use of a triple quadrupole mass spectrometer. The triple quadrupole mass spectrometer uses three quadrupoles in series. The first quadrupole (Q1) serves as a mass filter and selects for a subset of ions from the sample. The second quadrupole is non-mass filtering radio frequency only mass spectrometer. It serves as a collision cell and causes collision induced dissociation of the target ions from the mass filtering in Q1. Q3 is a second mass filtering step and provides a full analysis of the dissociated ions. One method of sample protein analysis in SRM involves the targeting a specific set of analyte peptides that correspond to the proteins of interest (Gallien, Duriez, & Domon, 2011). The samples are digested using a trypsin digest in order to give a set of peptides (Picotti & Aebersold, 2012). Isolation of the ions that correspond to the peptides from targeted proteins occurs in the selection step of Q1. This is followed by fragmentation in Q2. After fragmentation specific peptide fragments are selected for quantification and detection in Q3 (Gallien et al., 2011). The advantage of the SRM/MRM techniques is the ability to analyze specific target analytes within a complex background; a necessity in biological samples (Picotti & Aebersold, 2012). Selection of target peptides in the complex background common in biological samples is enhanced by the inclusion of stable isotope-labeled standard (SIS) peptides in the analysis (Percy, Chambers, Yang, Hardie, & Borchers, 2013). These SIS peptides are identically constructed copies of target peptides using specific heavy isotopes such as $^2$H or $^{13}$C. A known concentration of SIS peptides can be run with the matched peptide with an unknown concentration; comparison of the ratio of the intensity of the two peptides’ peaks allows for quantification of the unknown (Percy et al., 2013; Picotti & Aebersold, 2012). Running several different target peptides/ions sequentially and repeatedly allows for concurrent analysis of many target peptides at the same time from
the same sample (MRM) (Picotti & Aebersold, 2012). In a setup similar to the one utilized in this study, Percy et al. (2013), demonstrated that MRM is ideal as a rapid, highly specific, and comparatively inexpensive method to measure multiple blood plasma proteins for analysis as biomarkers from patients.

**LC System Parameters**

Peptide separations were achieved at 0.4 mL/min over a 60 min run, via a multi-step LC gradient (2-80% mobile phase B; mobile phase compositions: A was 0.1% FA in H2O while B was 0.1% FA in ACN). The column was maintained at 40°C. A post-gradient equilibration time of 4 min was used between each sample or standard analyzed.

**MRM acquisition parameters**

The MRM acquisition parameters employed were as follows: 3.5 kV capillary voltage, 300 V nozzle voltage, 11 L/min sheath gas flow at a temperature of 250°C, 15 L/min drying gas flow at a temperature of 150°C, 30 psi nebulizer gas pressure, 380 V fragmentor voltage, 5 V cell accelerator potential, and unit mass resolution in the first and third quadrupole mass analysers. The high energy dynode (HED) multiplier was set to -20 kV for improved ion detection efficiency and signal-to-noise ratios. In the quantitative analysis, the targets (1 transition/peptide) were monitored over 500 ms cycles and 1 min detection windows.

**MRM Data Analysis**

Data analysis involved peak inspection to ensure accurate selection, integration, and uniformity (in terms of peak shape and retention time) of the SIS and NAT peptide forms. After defining a small number of criteria (i.e., 1/x regression weighting, <20% deviation in the QC’s level’s accuracy) the standard curve was used to calculate the peptide concentration in fmol/ul of plasma in the patient samples through linear regression.

**Appendix N. Summary of PLAN Baseline, Deferiprone And Antioxidant Trials Biomarker Measurements**

**Supplementary Table 0-8.** Complete systemic biomarker results of a patient with PLAN at baseline and over the course of 37 weeks of deferiprone therapy.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Control* (n = 15)</th>
<th>PLAN Baseline (n = 1)</th>
<th>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein</td>
<td>0.097 ± 0.053</td>
<td>0.23</td>
<td>0.072</td>
</tr>
<tr>
<td>Malondialdehyde (uM)</td>
<td>2.27 ± 0.26</td>
<td>3.31</td>
<td>5.54</td>
</tr>
<tr>
<td>Free Malondialdehyde (uM)</td>
<td>0.45 ± 0.054</td>
<td>0.05</td>
<td>0.35</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>4.74</td>
<td>3.024</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.83 ± 0.22</td>
<td>6.75</td>
<td>3.57</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.33 ± 0.35</td>
<td>39.30</td>
<td>16.10</td>
</tr>
<tr>
<td>Adiponectin (fmol/ul)</td>
<td>69.36 ± 6.34</td>
<td>136.55</td>
<td>90.608</td>
</tr>
<tr>
<td>Alpha1-Antitrypsin (fmol/ul)</td>
<td>22090 ± 865.95</td>
<td>26714</td>
<td>19853</td>
</tr>
<tr>
<td>Anti-Thrombin III (fmol/ul)</td>
<td>23980 ± 818.93</td>
<td>24552</td>
<td>22511</td>
</tr>
<tr>
<td>Apolipoprotein Al (fmol/ul)</td>
<td>41320 ± 1466.18</td>
<td>27175</td>
<td>25751</td>
</tr>
<tr>
<td>Apolipoprotein AlV (fmol/ul)</td>
<td>974.1 ± 86.04</td>
<td>875.94</td>
<td>1265.1</td>
</tr>
<tr>
<td>Apolipoprotein B100 (fmol/ul)</td>
<td>124.6 ± 10.74</td>
<td>113.25</td>
<td>114.42</td>
</tr>
<tr>
<td>Apolipoprotein CI (fmol/ul)</td>
<td>5088 ± 320.73</td>
<td>3074.4</td>
<td>3812.5</td>
</tr>
<tr>
<td>Apolipoprotein CII (fmol/ul)</td>
<td>1070 ± 71.90</td>
<td>810.37</td>
<td>824.17</td>
</tr>
<tr>
<td>Apolipoprotein CIII (fmol/ul)</td>
<td>3893 ± 314.74</td>
<td>3366</td>
<td>3499</td>
</tr>
<tr>
<td>Apolipoprotein CIV (fmol/ul)</td>
<td>55.35 ± 7.29</td>
<td>64.296</td>
<td>60.306</td>
</tr>
<tr>
<td>Apolipoprotein D (fmol/ul)</td>
<td>1189 ± 59.15</td>
<td>1271.9</td>
<td>965.52</td>
</tr>
<tr>
<td>Apolipoprotein E (fmol/ul)</td>
<td>666.2 ± 56.18</td>
<td>874.56</td>
<td>604.08</td>
</tr>
<tr>
<td>Apolipoprotein F (fmol/ul)</td>
<td>162.4 ± 7.388</td>
<td>162.95</td>
<td>103.31</td>
</tr>
<tr>
<td>Apolipoprotein L1 (fmol/ul)</td>
<td>395.1 ± 42.14</td>
<td>344.63</td>
<td>352.6</td>
</tr>
<tr>
<td>Apolipoprotein M (fmol/ul)</td>
<td>288.4 ± 13.11</td>
<td>225.17</td>
<td>197</td>
</tr>
<tr>
<td>Apolipoprotein a (fmol/ul)</td>
<td>244.3 ± 95.57</td>
<td>61.717</td>
<td></td>
</tr>
<tr>
<td>Cholinesterase (fmol/ul)</td>
<td>37.32 ± 2.34</td>
<td>51.55</td>
<td>35.16</td>
</tr>
<tr>
<td>Marker</td>
<td>Control* (n = 15)</td>
<td>PLAN Baseline (n = 1)</td>
<td>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------</td>
<td>----------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Fibrinogen Alpha Chain (fmol/ul)</td>
<td>5759 ± 340.5</td>
<td>10724</td>
<td>5393.5</td>
</tr>
<tr>
<td>Fibrinogen Beta Chain (fmol/ul)</td>
<td>14480±728.06</td>
<td>24534</td>
<td>13576</td>
</tr>
<tr>
<td>Fibrinogen Gamma Chain (fmol/ul)</td>
<td>5178 ± 221.11</td>
<td>8670.4</td>
<td>4664.1</td>
</tr>
<tr>
<td>Glutathione Peroxidase 3 (fmol/ul)</td>
<td>92.95 ± 4.51</td>
<td>124.88</td>
<td>90.65</td>
</tr>
<tr>
<td>Neuropilin-2 (fmol/ul)</td>
<td>33.15 ± 1.08</td>
<td>27.94</td>
<td>29.28</td>
</tr>
<tr>
<td>Phospholipid Transfer Protein (fmol/ul)</td>
<td>42.82 ± 2.22</td>
<td>47.784</td>
<td>33.944</td>
</tr>
<tr>
<td>Retinol-Binding Protein 4 (fmol/ul)</td>
<td>699.2 ± 44.46</td>
<td>654</td>
<td>712.48</td>
</tr>
<tr>
<td>Serum Albumin (fmol/ul)</td>
<td>412100 ± 11461.9</td>
<td>386880</td>
<td>336240</td>
</tr>
<tr>
<td>Serum Amyloid A-1 and A-2 Proteins (fmol/ul)</td>
<td>78.98 ± 27.88</td>
<td>56.976</td>
<td>48.88</td>
</tr>
<tr>
<td>Serum Amyloid A-4 Protein (fmol/ul)</td>
<td>1211 ± 121.28</td>
<td>1264.6</td>
<td>732.21</td>
</tr>
<tr>
<td>Serum Amyloid P-Component (fmol/ul)</td>
<td>577.7 ± 47.12</td>
<td>1049.8</td>
<td>537.04</td>
</tr>
<tr>
<td>Sex Hormone-Binding Globulin (fmol/ul)</td>
<td>63.85 ± 7.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transthyretin (fmol/ul)</td>
<td>476.8 ± 42.76</td>
<td>226.42</td>
<td>451.03</td>
</tr>
<tr>
<td>Vitamin D-Binding Protein (fmol/ul)</td>
<td>4177 ± 121.622</td>
<td>4119.3</td>
<td>3652.3</td>
</tr>
<tr>
<td>Zinc Alpha 2-Glycoprotein (fmol/ul)</td>
<td>615.8 ± 25.61</td>
<td>732.23</td>
<td>613.87</td>
</tr>
</tbody>
</table>

*All control values are ± SE
Supplementary Table 0-9. Complete systemic biomarker results of a patient with PLAN at baseline and over the course of 20 months of antioxidant therapy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control*</th>
<th>PLAN Pre-Antioxidant Trial</th>
<th>PLAN End of Antioxidant Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein</td>
<td>0.098 ± 0.054</td>
<td>0.026</td>
<td>0.94</td>
</tr>
<tr>
<td>Malondialdehyde (uM)</td>
<td>2.27 ± 0.27</td>
<td>4.06</td>
<td>6.87</td>
</tr>
<tr>
<td>Free Malondialdehyde (uM)</td>
<td>0.45 ± 0.054</td>
<td>0.68</td>
<td>0.55</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>4.98</td>
<td>3.27</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.84 ± 0.22</td>
<td>6.37</td>
<td>3.24</td>
</tr>
<tr>
<td>TNFα (pg/mL)</td>
<td>2.33 ± 0.36</td>
<td>47.16</td>
<td>14.27</td>
</tr>
<tr>
<td>Adiponectin (fmol/μL)</td>
<td>69.36 ± 6.34</td>
<td>158.4</td>
<td>179.8</td>
</tr>
<tr>
<td>Alpha1-Antitrypsin (fmol/μL)</td>
<td>22,090 ± 865.95</td>
<td>21,739</td>
<td>31,837</td>
</tr>
<tr>
<td>Anti-Thrombin III (fmol/μL)</td>
<td>23,980 ± 818.93</td>
<td>24,510</td>
<td>31,407</td>
</tr>
<tr>
<td>Apolipoprotein AI (fmol/μL)</td>
<td>41,320 ± 1466.18</td>
<td>31,426.0</td>
<td>35,492.0</td>
</tr>
<tr>
<td>Apolipoprotein AIV (fmol/μL)</td>
<td>974.1 ± 86.04</td>
<td>1,249.0</td>
<td>1,095.1</td>
</tr>
<tr>
<td>Apolipoprotein B100 (fmol/μL)</td>
<td>124.6 ± 1.074</td>
<td>137.0</td>
<td>170.8</td>
</tr>
<tr>
<td>Apolipoprotein CI (fmol/μL)</td>
<td>5088 ± 320.73</td>
<td>5,883.0</td>
<td>6,922.1</td>
</tr>
<tr>
<td>Apolipoprotein CII (fmol/μL)</td>
<td>1070 ± 71.90</td>
<td>899.3</td>
<td>1,327.2</td>
</tr>
<tr>
<td>Apolipoprotein CIII (fmol/μL)</td>
<td>3893 ± 314.74</td>
<td>3,441.7</td>
<td>4,666.7</td>
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<tr>
<td>Apolipoprotein CIV (fmol/μL)</td>
<td>55.35 ± 7.29</td>
<td>35.6</td>
<td>90.5</td>
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<tr>
<td>Apolipoprotein D (fmol/μL)</td>
<td>1189 ± 59.15</td>
<td>1,276.8</td>
<td>1,646.9</td>
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<tr>
<td>Apolipoprotein E (fmol/μL)</td>
<td>666.2 ± 56.18</td>
<td>897.5</td>
<td>945.3</td>
</tr>
<tr>
<td>Apolipoprotein F (fmol/μL)</td>
<td>162.4 ± 7.39</td>
<td>128.8</td>
<td>215.9</td>
</tr>
<tr>
<td>Apolipoprotein L1 (fmol/μL)</td>
<td>395.1 ± 42.14</td>
<td>310.6</td>
<td>419.9</td>
</tr>
<tr>
<td>Apolipoprotein M (fmol/μL)</td>
<td>288.4 ± 13.11</td>
<td>215.0</td>
<td>289.1</td>
</tr>
<tr>
<td>Cholinesterase (fmol/μL)</td>
<td>37.32 ± 2.34</td>
<td>33.8</td>
<td>45.6</td>
</tr>
<tr>
<td>Marker</td>
<td>Control* <em>(n = 15)</em></td>
<td>PLAN Baseline <em>(n = 1)</em></td>
<td>PLAN 37 Weeks of Deferiprone Therapy <em>(n = 1)</em></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------------------</td>
<td>-------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Fibrinogen Alpha Chain (fmol/uL)</td>
<td>5759 ± 340.5</td>
<td>5,133.1</td>
<td>8,661.3</td>
</tr>
<tr>
<td>Fibrinogen Beta Chain (fmol/uL)</td>
<td>14,480 ± 728.06</td>
<td>14,217.0</td>
<td>20,002.0</td>
</tr>
<tr>
<td>Fibrinogen Gamma Chain (fmol/uL)</td>
<td>5178 ± 221.11</td>
<td>5,135.5</td>
<td>7,220.2</td>
</tr>
<tr>
<td>Glutathione Peroxidase 3 (fmol/uL)</td>
<td>92.95 ± 4.51</td>
<td>109.2</td>
<td>123.6</td>
</tr>
<tr>
<td>Neuropilin-2 (fmol/uL)</td>
<td>33.15 ± 1.08</td>
<td>109.2</td>
<td>123.6</td>
</tr>
<tr>
<td>Phospholipid Transfer Protein (fmol/uL)</td>
<td>42.82 ± 2.22</td>
<td>34.9</td>
<td>35.0</td>
</tr>
<tr>
<td>Retinol-Binding Protein 4 (fmol/uL)</td>
<td>699.2 ± 44.46</td>
<td>764.7</td>
<td>772.7</td>
</tr>
<tr>
<td>Serum Albumin (fmol/uL)</td>
<td>412,100 ± 11,461.9</td>
<td>325,690.0</td>
<td>367,590.0</td>
</tr>
<tr>
<td>Serum Amyloid A-1 and A-2 Proteins (fmol/uL)</td>
<td>78.98 ± 27.88</td>
<td>30.4</td>
<td>172.8</td>
</tr>
<tr>
<td>Serum Amyloid A-4 Protein (fmol/uL)</td>
<td>1211 ± 121.280</td>
<td>1,780.7</td>
<td>2,721.5</td>
</tr>
<tr>
<td>Serum Amyloid P-Component (fmol/uL)</td>
<td>577.7 ± 47.12</td>
<td>683.0</td>
<td>1,240.8</td>
</tr>
<tr>
<td>Transthyretin (fmol/uL)</td>
<td>476.8 ± 42.76</td>
<td>226.42</td>
<td>451.03</td>
</tr>
<tr>
<td>Vitamin D-Binding Protein (fmol/uL)</td>
<td>4177 ± 121.62</td>
<td>3,737.0</td>
<td>5,245.4</td>
</tr>
<tr>
<td>Zinc Alpha 2-Glycoprotein (fmol/uL)</td>
<td>615.8 ± 25.611</td>
<td>752.2</td>
<td>870.7</td>
</tr>
</tbody>
</table>
Appendix O. PKAN Deferiprone Study Sample Collection Overview

TIRCON and the UCSF Benioff Children’s Hospital PKAN Deferiprone Trial
Biomarker Substudy Overview

I. Sample Collection

Study sample collection occurred as a substudy of the ongoing deferiprone trial. Samples were collected at scheduled patient visits for the main trial at baseline, 6 months, 12 months and 18 months (Green). Time points which occurred prior to the start of our substudy or which were missed by the patient did not have any sample collection (Red).

Sample Totals:

6 Patients at Baseline
5 Patients at 6 Months of Deferiprone Therapy
11 Patients at 12 Months of Deferiprone Therapy
24 Patients at 18 Months of Deferiprone Therapy

Due to the later start of the substudy limited patient numbers were available at early time points in the study.

II. TIRCON Biobank

Additional Samples (Blue) were collected from the main study biobank and used to fill in some of the missing time points. These samples were prioritize for inclusion in the proteomic analysis. This is the cause of the difference between the baseline proteomic group and baseline main study group.

Sample Totals:

24 Patients at 18 Months + 4 = 10 at Baseline
6 Patients at Baseline + 6 = 11 at 6 Months
5 Patients at 6 Months + 14 = 25 at 12 Months
11 Patients at 12 Months = 24 at 18 Months

Supplementary Figure 0-12. Outline of sample collection details for the biomarker substudy of the phase 3 double blinded, randomized, placebo controlled clinical trial of deferiprone therapy in patients with Pantothenate Kinase-Associated Neurodegeneration.
## Appendix P. Summary of PKAN Baseline and Deferiprone Trial Biomarker Measurements

### Supplementary Table 0-10.
Complete systemic biomarker results of a cohort of patients with Pantothenate Kinase Associated Neurodegeneration (PKAN) at baseline and over the course of 18 months of therapy with Deferiprone

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control* (n = 15)</th>
<th>PKAN Baseline (n = 16)</th>
<th>PKAN 18 Months of Deferiprone Therapy (n = 15)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein</td>
<td>0.097 ± 0.053</td>
<td>0.55 ± 0.18</td>
<td>1.31 ± 1.04</td>
<td>0.013</td>
</tr>
<tr>
<td>Malondialdehyde (uM)</td>
<td>2.27 ± 0.26</td>
<td>3.16 ± 0.33</td>
<td>2.98 ± 0.35</td>
<td>0.046</td>
</tr>
<tr>
<td>Free Malondialdehyde (uM)</td>
<td>0.45 ± 0.054</td>
<td>0.73 ± 0.093</td>
<td>0.522 ± 0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>2.44 ± 0.27</td>
<td>2.68 ± 0.28</td>
<td>3.364 ± 0.216</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>1.83 ± 0.22</td>
<td>3.025 ± 0.69</td>
<td>3.2 ± 0.66</td>
<td>0.068</td>
</tr>
<tr>
<td>TNFa (pg/mL)</td>
<td>2.33 ± 0.35</td>
<td>3.364 ± 0.98</td>
<td>4.71 ± 0.99</td>
<td>0.037</td>
</tr>
<tr>
<td>Adiponectin (fmol/uL)</td>
<td>69.36 ± 6.34</td>
<td>106.4±11.82</td>
<td>95.33 ± 8.68</td>
<td>0.011</td>
</tr>
<tr>
<td>Alpha1-Antitrypsin (fmol/uL)</td>
<td>22090 ± 865.95</td>
<td>23510 ± 1395.09</td>
<td>23840 ± 1152.97</td>
<td></td>
</tr>
<tr>
<td>AntiThrombin III (fmol/uL)</td>
<td>23980 ± 818.93</td>
<td>24740±82.97</td>
<td>25140 ± 778.89</td>
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</tr>
<tr>
<td>Apolipoprotein Al (fmol/uL)</td>
<td>41320 ± 1466.18</td>
<td>37560 ± 1179.57</td>
<td>36500 ± 1312.94</td>
<td>0.053</td>
</tr>
<tr>
<td>Apolipoprotein AIV (fmol/uL)</td>
<td>974.1 ± 86.04</td>
<td>934 ± 71.72</td>
<td>894.8 ± 88.89</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein B100 (fmol/uL)</td>
<td>124.6 ± 10.74</td>
<td>94.9 ± 14.25</td>
<td>147.8 ± 13.94</td>
<td>0.10</td>
</tr>
<tr>
<td>Apolipoprotein CI (fmol/uL)</td>
<td>5088 ± 320.73</td>
<td>4083 ± 474.98</td>
<td>5412 ± 313.39</td>
<td>0.0914</td>
</tr>
<tr>
<td>Apolipoprotein CII (fmol/uL)</td>
<td>1070 ± 71.90</td>
<td>918.6 ± 94.12</td>
<td>1183 ± 91.39</td>
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<tr>
<td>Apolipoprotein CIII (fmol/uL)</td>
<td>3893 ± 314.74</td>
<td>3284 ± 253.82</td>
<td>4100 ± 245.02</td>
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</tr>
<tr>
<td>Marker</td>
<td>Control* (n = 15)</td>
<td>PLAN Baseline (n = 1)</td>
<td>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</td>
<td>Significance Control vs Baseline</td>
</tr>
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<td>-------------------</td>
<td>-----------------------</td>
<td>---------------------------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Apolipoprotein CIV (fmol/uL)</td>
<td>55.35±7.29</td>
<td>37.99±4.65</td>
<td>55.08±6.02</td>
<td>0.056</td>
</tr>
<tr>
<td>Apolipoprotein D (fmol/uL)</td>
<td>1189 ± 59.15</td>
<td>1210 ± 144.64</td>
<td>960.6 ± 90.35</td>
<td>0.045</td>
</tr>
<tr>
<td>Apolipoprotein E (fmol/uL)</td>
<td>666.2 ± 56.18</td>
<td>692.9 ± 41.25</td>
<td>661.2 ± 49.99</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein F (fmol/uL)</td>
<td>162.4 ± 7.388</td>
<td>168.3 ± 18.57</td>
<td>138.6 ± 9.05</td>
<td>0.05134</td>
</tr>
<tr>
<td>Apolipoprotein L1 (fmol/uL)</td>
<td>395.1 ± 42.14</td>
<td>358.1±32.7</td>
<td>385.9 ± 35.73</td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein M (fmol/uL)</td>
<td>288.4 ± 13.11</td>
<td>294.8 ± 11.77</td>
<td>288.6 ± 17.52</td>
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</tr>
<tr>
<td>Apolipoprotein a (fmol/uL)</td>
<td>244.3 ± 95.57</td>
<td>360.9 ± 75.9</td>
<td>241.4 ± 62.2</td>
<td></td>
</tr>
<tr>
<td>Cholinesterase (fmol/uL)</td>
<td>37.32 ± 2.34</td>
<td>42.25 ± 3.19</td>
<td>46 ± 2.2</td>
<td>0.011</td>
</tr>
<tr>
<td>Fibrinogen alpha chain (fmol/uL)</td>
<td>5759 ± 340.5</td>
<td>9481 ± 723.17</td>
<td>6215 ± 411.76</td>
<td>0.00013</td>
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<tr>
<td>Fibrinogen beta chain (fmol/uL)</td>
<td>14480±72.86</td>
<td>23440±1801.83</td>
<td>16040 ± 1301.42</td>
<td>0.00018</td>
</tr>
<tr>
<td>Fibrinogen gamma chain (fmol/uL)</td>
<td>5178 ± 221.11</td>
<td>8511 ± 601.87</td>
<td>5839 ± 462.33</td>
<td>5.16E-05</td>
</tr>
<tr>
<td>Glutathione peroxidase 3 (fmol/uL)</td>
<td>92.95 ± 4.51</td>
<td>79.18±5.5</td>
<td>82.59±4.387</td>
<td>0.021</td>
</tr>
<tr>
<td>Neuropilin-2 (fmol/uL)</td>
<td>33.15 ± 1.08</td>
<td>30.85±1.19</td>
<td>33.94 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Phospholipid transfer protein (fmol/uL)</td>
<td>42.82 ± 2.22</td>
<td>35.43 ± 2.45</td>
<td>37.3 ± 2.71</td>
<td>0.033</td>
</tr>
<tr>
<td>Retinol-binding protein 4 (fmol/uL)</td>
<td>699.2 ± 44.46</td>
<td>547 ± 41.19</td>
<td>722.6 ± 50.96</td>
<td>0.018</td>
</tr>
<tr>
<td>Serum albumin (fmol/uL)</td>
<td>412100 ± 11461.9</td>
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<td>407900 ± 10485.4</td>
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</tr>
<tr>
<td>Marker</td>
<td>Control* (n = 15)</td>
<td>PLAN Baseline (n = 1)</td>
<td>PLAN 37 Weeks of Deferiprone Therapy (n = 1)</td>
<td>Significance</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------</td>
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<td>-------------------------------------------</td>
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<tr>
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<td>Control vs Baseline</td>
</tr>
<tr>
<td>Serum amyloid A-1 and A-2 proteins (fmol/uL)</td>
<td>78.98 ± 27.88</td>
<td>97.67 ± 25.8</td>
<td>669.7 ± 577.13</td>
<td>0.093</td>
</tr>
<tr>
<td>Serum amyloid A-4 protein (fmol/uL)</td>
<td>1211 ± 121.28</td>
<td>1601 ± 249.03</td>
<td>1407 ± 224.28</td>
<td></td>
</tr>
<tr>
<td>Serum amyloid P-component (fmol/uL)</td>
<td>577.7 ± 47.12</td>
<td>444.5 ± 50.08</td>
<td>725.2 ± 74.01</td>
<td>0.063</td>
</tr>
<tr>
<td>Sex hormone-binding globulin (fmol/uL)</td>
<td>63.85 ± 7.57</td>
<td>89.43 ± 13.02</td>
<td>83.25 ± 9.35</td>
<td>0.105</td>
</tr>
<tr>
<td>Transthyretin (fmol/uL)</td>
<td>476.8 ± 42.76</td>
<td>320.4 ± 30.49</td>
<td>463.8 ± 59.9</td>
<td>0.0063</td>
</tr>
<tr>
<td>Vitamin D-binding protein (fmol/uL)</td>
<td>4177 ± 121.622</td>
<td>4045 ± 165.84</td>
<td>4408 ± 197.12</td>
<td></td>
</tr>
<tr>
<td>Zinc-alpha-2-glycoprotein (fmol/uL)</td>
<td>615.8 ± 25.61</td>
<td>518.1 ± 36.71</td>
<td>590.6 ± 29.26</td>
<td>0.0381</td>
</tr>
</tbody>
</table>
Appendix Q Additional PKAN Study Figures

**Supplementary Figure 0-13.** Intrarelationships between plasma proteins correlating with disease severity. The plasma proteins transthyretin, fibrinogen alpha chain zinc alpha 2-glycoprotein and retinol-binding protein were measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN, n = 16). A significant negative correlation was seen between transthyretin and fibrinogen alpha chain, $p = 0.049$ (A) as well as zinc alpha 2-glycoprotein, $p = 0.1$ (C) was observed in PKAN patients (Pearson’s correlation). A significant positive correlation was seen between zinc alpha 2-glycoprotein and fibrinogen alpha chain, $p = 0.003$ (B) as well reintro-binding protein, $p<0.001$ (D) was observed in PKAN patients (Pearson’s correlation).
Supplementary Figure 0-14. Plasma expression of the apolipoproteins CI (A), CII (B), CIV (C) and B100 (D) in patients with Pantothenate Kinase Associated Neurodegeneration (PKAN) at baseline and following 18 months of deferiprone (DFP) therapy (n = 16 at baseline and n = 15 at 18 months) relative to controls (n = 15); as measured by UVic Genome BC Proteomic Center multiple reaction monitoring mass spectrometry proteomics. Line = median; box = 25th to 75th percentiles, bars = 5th to 95th percentiles. Plasma apolipoprotein levels were significantly elevated in patients following 18 months of deferiprone therapy compared to baseline, p =0.028, p = 0.028, p = 0.033 and p = 0.012 (*, 2-tailed, unpaired, students t-test).