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Exogenous ketones as a metabolic intervention for Parkinson's disease

Doctor of Philosophy Thesis

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Declaration

I understand the University of Oxford's policy on plagiarism and certify that this thesis represents my own work, except where indicated by appropriate references. I further certify the work presented in this thesis has not been submitted in support of another degree or qualification from the University of Oxford or another institute.

Acknowledgements

This work, along with the education and overall life experience I have been privileged to receive at the University of Oxford, would not have been possible without the mentorship of my supervisor, Professor Kieran Clarke. The combination of confidence she expressed in my abilities, opportunities she presented to me, and freedom she allowed me to pursue my own ideas enabled me to make the most out of my academic experience.

Additionally, I would like to acknowledge my senior peer, Dr. Luis Adrian Soto Mota, for his eagerness to discuss new ideas and collaborate on projects, and for his friendship. Dr. Mota, together with my family, supported me through my personal health struggles, an invaluable contribution for which I will be forever grateful.

My time at Oxford was fully funded by the Keasbey Memorial Foundation Scholarship. In the tradition of the scholarship, established by Miss Marguerite A. Keasbey, I would lastly like to remember her father, Henry G. Keasbey.

COVID-19 statement

The advent of the COVID-19 pandemic necessitated a suspension of the last of my three clinical studies in Parkinson disease. I completed two of the studies as well as study design, ethics, contracts, and four participants for the third. As it was not possible to complete the final study, the Department of Physiology, Anatomy and Genetics agreed that it would be reasonable for me to submit a thesis composed of my two complete studies and my one incomplete study as a pilot and validation of methods, supplemented with my recent published peer-reviewed manuscripts and an introduction to a technology that I am developing to assist future research in nutrition.

Therefore, this DPhil thesis is broad in scope and unconventional in composition. It includes original research in the form of two completed clinical studies, one pilot study, one case report, and one technological development; and it includes further intellectual contributions in the form of published reviews and theory papers. Its contents should collectively demonstrate my contributions to the fields of ketogenics and neurodegenerative and neurological diseases as well as my competence as a clinical researcher and scientific writer.

Abstract

Neurodegenerative and neurological diseases are metabolic diseases that invite metabolic treatments. Nutritional interventions that induce a state of ketosis, or "ketogenics," are one class of metabolic treatment that have the potential to address the root pathologies of such diseases. Presented first in this thesis are three clinical studies that investigated the effects of a ketone ester (KE) drink in patients with Parkinson's disease. The first study was a randomized, placebo-controlled, crossover trial in 14 participants that demonstrated a significant improvement in endurance exercise performance following a KE drink. Participants sustained a therapeutic cycling cadence of 80 rpm for 24% longer after ingesting KE as compared to performance following a placebo (carbohydrate) drink. The second study utilized ^{31}P magnetic resonance spectroscopy in 11 participants and demonstrated that exogenous KE did not alter brain energetics one hour following a drink. The third study was a randomized, placebo-controlled 28-day intervention in which 20 participants were to ingest either KE or placebo drinks four times daily for one month, while motor and non-motor symptoms were monitored. Four participants completed the protocol before the COVID-19 pandemic stopped Oxford clinical studies. Thus, the completed work is included herein as a validation of methods and feasibility study. This thesis also includes intellectual contributions in the form of recent peer-reviewed publications, most of which are literature reviews and theoretical papers, although one is a case report and another is a technological innovation: a tracking tool that has potential to open new avenues in nutrition research. Although the contents of this thesis may appear disparate, they are woven around the narrative that brain disorders are caused by disturbances in metabolism, and that the development of disease-modifying therapies, including ketogenics, will likely require addressing the core metabolic dysfunction that underlies each disease.

Abbreviations

Aβ, amyloid β; **ACAT**, acetyl-coenzyme A acetyltransferases; **AD**, Alzheimer's disease; **ADAM10**, A disintegrin and metalloproteinase domain-containing protein 10; **ADHD**, attention deficient hyperactivity disorder; **AGE**, advanced glycation end product; **AICD**, APP intracellular domain; **Akt**, protein kinase B; **ALA**, alpha-linolenic acid; **ALS**, amyotrophic lateral sclerosis; **AMPK**, AMP-activated protein kinase; **APN**, adiponectin; **Apo(B)**, apolipoprotein B; ***ApoE4***, apolipoprotein E4 gene; **APP**, amyloid precursor protein; **ASD**, autism spectrum disorder; **ATGs**, *autophagy*-related proteins; **ATP**, adenosine triphosphate; **A2ATP13A2**, ATPase cation transporting 13A2; ***BACE1***, beta-secretase 1 gene; **BAD**, Bcl2 associated agonist of cell death; **BDNF**, brain-derived neurotrophic factor; **BED**, binge eating disorder; **βHB**, D-β-hydroxybutyrate; **BPD**, bipolar disorder; **CBP**, CREB-binding protein; **CD36**, cluster of differentiation 36; **cGM**, cerebral glucose metabolism; **CHCHD2**, coiled-coil-helix-coiled-coil-helix domain containing 2; **CREB**, cAMP response element-binding protein; **CRP**, C-reactive protein; **CSF**, cerebrospinal fluid; **CVD**, cardiovascular disease; **C9orf72**; chromosome 9 open reading frame 72; **Deptor**, domain-containing mTOR-interacting protein; **DHA**, docosahexaenoic acid; **DJ-1**, PARK7; **DKK1**, Dickkopf 1; **DVL**, Disheveled; **EPA**, eicosapentaenoic acid; **ER**, endoplasmic reticulum; **FAD**, flavin adenine dinucleotide; **FBXO7**, F-box only protein 7; **FDA**, Food and Drug Administration; **FFAR**, free fatty acid receptor; **FOXO3A**, forkhead box O3A; **FSS**, fatigue severity survey; **FTD**, frontotemporal dementia; **Fz**, Frizzled; **GABA**, gamma-aminobutyric acid; **GBA**, glucocerebrosidase; **GDNF**, glial-derived neurotrophic factor; **GLP-1**, glucagon-like peptide 1; **GPC**, glycerophosphocholine; **GPE**, glycerophosphoethanolamine; **GSK3β**, glycogen synthase kinase 3β; **HCAR2**, hydroxycarboxylic acid receptor 2; **HD**, Huntington's disease; **HDAC**, class I/II histone deacetylase; **HDL-C**, HDL content of HDL particles; **HDL-P**, HDL particle count; **HHLA-DR**, human leukocyte antigen-DR isotype; **HMG**, 3-hydroxymethylglutaryl; **HRA**, Health Research Authority; **hsCRP**, high-sensitivity C-reactive protein; **IBD**, inflammatory bowel

disease; **IBS**, irritable bowel syndrome; **IDE**, insulin degrading enzyme; **IL-1β**, interleukin-1β; **iNOS**, inducible nitric oxide synthase; **IR**, insulin receptor; **IRAS**, integrated research application system; **IRS**, insulin receptor substrate; **ISRCTN**, international standard randomized controlled trial serial number; **KD**, ketogenic diet; **KE,** ketone ester, (R)-3-hydroxybutyl-(R)-3-hydroxybutyrate; **LDL-C**, LDL content of LDL particles; **LDL-P**, LDL particle count; **LEDD**, levodopa equivalent daily dose**;** **Lp(a)**, lipoprotein(a); **LP-PLA2**, lipoprotein-associated phospholipase A2; **LPS**, lipopolysaccharide; **LRP-6**, low-density lipoprotein receptor-related protein 6; **LRRK2/PARK8**, leucine-rich repeat kinase 2; ***MAPT***, tau gene; **MCI** mild cognitive impairment; **MCT**, monocarboxylate transporters; **MDD**, major depressive disorder; **MDS-UPDRS**, Movement Disorder Society Unified Parkinson's Disease Rating Scale; **MHC**; major histocompatibility complex; **mGluR5**, metabotropic glutamate receptor type 5; **mLST8**, mammalian lethal with SEC13 protein 8; **mnSOD**, manganese superoxide dismutase; **MoCA**, Montreal Cognitive Assessment; **MPP^+**, 1-methyl-4-phenylpyridinium, **MPTP**, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; **MS**, multiple sclerosis; **MSFD2A**, major facilitator superfamily domain-containing protein 2 A; ***MTHFR***, methylenetetrahydrofolate reductase gene; **mTOR**, mechanistic target of rapamycin; **Mt2**, metallothionein 2; **MUNCH**, My Ultimate Nutrition Calculator and Helper; **NAD**, nicotinamide adenine dinucleotide; **NADP***,* nicotinamide adenine dinucleotide phosphate; **NAMPT**, nicotinamide phosphoribosyltransferase; **NFκB**, nuclear factor κ-light-chain-enhancer of activated B cells; **NFT**, neurofibrillary tangle; **NHS**, National Health Service; **NLRP3**, NOD-, LRR- and pyrin domain-containing protein 3; **NO**, nitric oxide; **NOX2**, NADPH oxidase; **NPY**, neuropeptide Y; **OMCR**, Oxford Center for Clinical Magnetic Resonance Research; **OE**, oxygen economy; **OPDC**, Oxford Parkinson's Disease Centre; **oxLDL**, oxidized LDL; **PARP1**, poly ADP-ribose polymerase 1; **PCr**, phosphocreatine; **PD**, Parkinson's disease; **PDK-1**, phosphoinositide-dependent kinase-1; **PDQ-Carer**, Parkinson's disease questionnaire for carers; **PDQ-39**, Parkinson's disease questionnaire-39; **PDT**, personal digital therapeutic; **PGC1α**,

peroxisome proliferator-activated receptor gamma coactivator 1 α; **Pi**, inorganic phosphate; **PINK1**, PTEN-induced kinase 1; **PIP3**, phosphatidylinositol; **PI3K**, phosphoinositide 3 kinase; **PLA2G6**, phospholipase A2 group 6; **PP**, pancreatic polypeptide; **PPAR**; peroxisome proliferator-activated receptor; **PRAS40**, proline-rich Akt substrate of 40kDa; **PTEN**, phosphatase and tensin homolog; **PTSD**, post-traumatic stress disorder; **p53**, tumor suppressor p53; **p70S6K1**, p70 ribosomal S6 protein kinase 1; **Q**, coenzyme Q; **Raptor**, regulatory-associated protein of mTOR; **RBD**, rapid eye movement sleep behavior disorder; **RBDSQ**, RBD screening questionnaire; **REC**, research ethics committee; **REDCap**, Research Electronic Data Capture; **REM**, rapid eye movement; **RER**, respiratory exchange ratio; **Rheb**, Ras homolog enriched in brain protein; **ROS**, reactive oxygen species; **RPE**, relative perceived exertion; **rpm**, revolutions per minute; **RQ**, respiratory quotient; **SAD**, Standard American Diet; **SCFA**, short chain fatty acid; **SCOT**, succinyl-CoA-3-oxaloacid CoA transferase; **SIRT**, silent mating type information regulation 2 homolog; **SMCR8**, Smith-Magenis syndrome chromosome region 8; **SN**, substantia nigra; ***SNCA*****,** α-synuclein gene; **SPARX**, Study in Parkinson's Disease of Exercise; **SZ**, schizophrenia; **TFEB**, transcription factor EB; **TLR**, toll-like receptor; **TNFα**, tumor necrosis factor α; **TSC1/2**, Tuberous sclerosis protein-complex; **TSPO**, translocator protein; **T2DM**, type 2 diabetes mellitus; ***UBQLN2/4***, ubiquilin genes; **ULK1**, unc-51 like autophagy activating kinase 1; **VCO_2**, carbon dioxide expiration rate; **V_E**, ventilation rate; **VEGF**, vascular endothelial growth factor; **VO_2**, oxygen consumption rate; **VO_{2Max}**, maximum oxygen consumption rate; **VO_{2Peak}**, peak oxygen consumption rate on endurance test; **VPS35**, vacuolar protein sorting-associated protein 35; **^{31}P-MRS**, 31-phosphorus-magnetic resonance spectroscopy; **4E-BP1**, 4E-binding protein 1; **6-OHDA**, 6-hydroxydopamine; **ΔG**, Gibbs free energy; **ΔG®**, (R)-3-hydroxybutyl (R)-3-hydroxybutyrate

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Chapter 1. *Introduction*

1.1 Ketone body metabolism

1.1.1 Evolutionary perspective

Homo sapiens' large, complex brains enabled the success of our species. But our large brains carry a heavy metabolic burden. Specifically, neurons principally rely on glucose oxidation. As such, the brain consumes 400 – 500 Calories per day, accounting for 60% of whole body glucose consumption in the resting state [1, 2]. From an evolutionary perspective, this is problematic. While a human's energy store of fat is on the order of 135,000 Calories, only about 450 Calories of glucose are stored in the form of liver glycogen to provide the brain with energy [3].

Given the limited stores of glucose available to the brain, carbohydrate deprivation requires the catabolism of lean tissue for gluconeogenesis. Were the human brain solely reliant on gluconeogenesis, the average human would not be able to survive much longer than two weeks without food. In reality, humans can survive about two months of starvation [2]. The ability to survive longer periods of fasting and famine is courtesy of the evolutionary advent of ketone bodies: alternative fuel substrates made by the liver from fat tissue during periods of carbohydrate scarcity. The principle ketone bodies found in the blood are D-β-hydroxybutyrate (βHB) and acetoacetate.

George Cahill and his colleagues were the first to propose and demonstrate, in the 1960s and 1970s, that when subjects were subjected to prolonged fasting, levels of serum ketone bodies increased, rising from close to zero to ~1-2 mmol/L within a couple of days, and gradually increasing over weeks to ~6 – 8 mmol/L in order to supply the brain and extra-hepatic peripheral tissues with fuel [4-6] (Figure 1.1). Without ketone bodies, it is unlikely that our species would have survived.

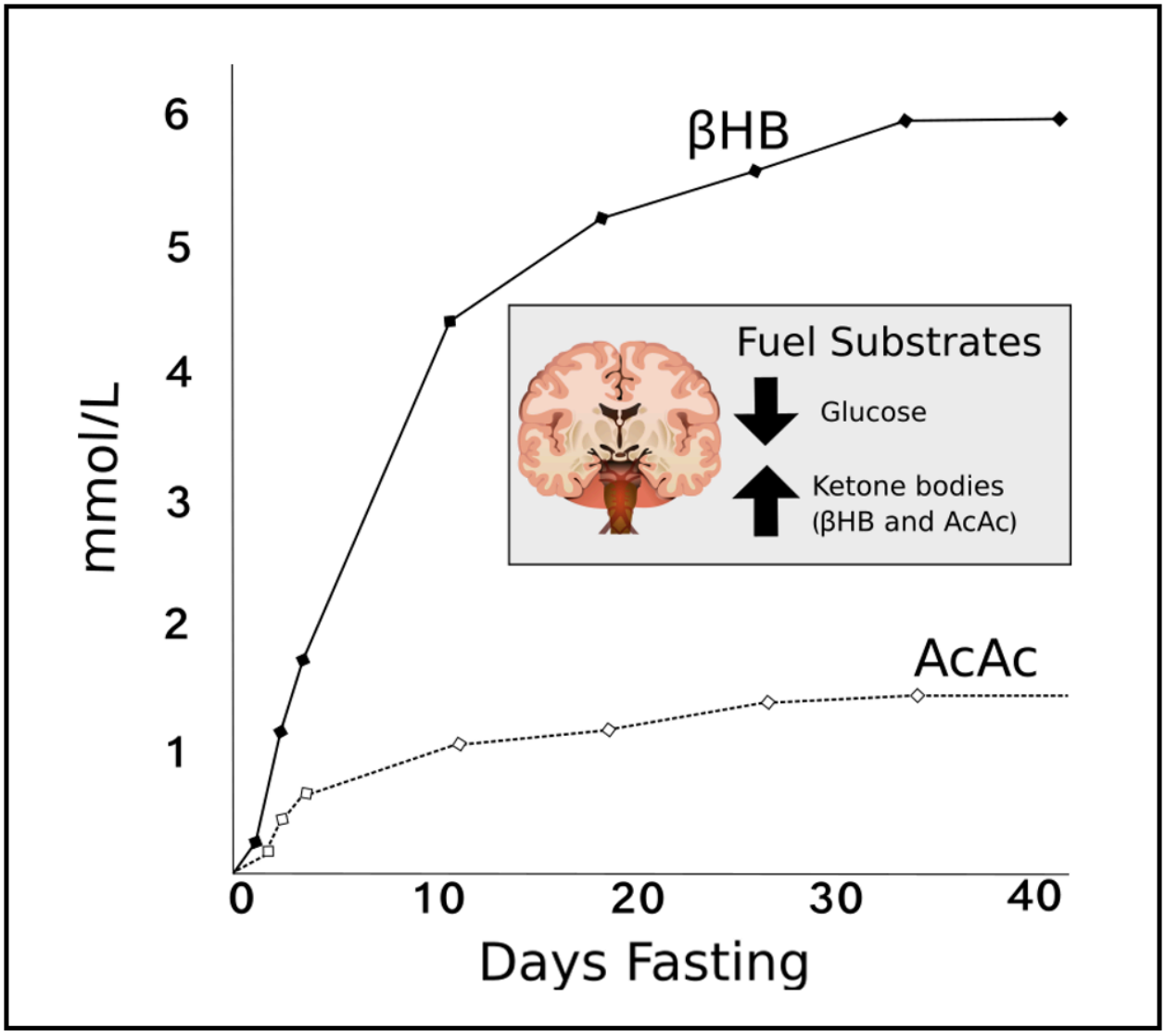


Figure 1.1 Fasting induces ketosis and ketone bodies fuel the brain

In fasted man, blood levels of the ketone bodies, β-hydroxybutyrate (βHB) and acetoacetate (AcAc), gradually increase. The brain utilizes these ketone bodies as fuel to spare glucose and thus preserve gluconeogenic lean tissues, such as skeletal muscles, from catabolism during starvation. (This figure was adapted from [6], based on work originally performed by George Cahill and colleagues [4].)

1.1.2 Endogenous production

Starvation and fasting induce the liberation of fatty acids from adipose reserves into circulation, from where they can be taken up by hepatocytes and converted into the ketone body, βHB. Once inside mitochondria, fatty acids undergo β-oxidation to acetyl-CoA. Rather than entering the Krebs cycle to be completely oxidized to carbon dioxide, the enzyme acetyl-CoA acetyltransferase (ACAT) condenses two acetyl-CoA molecules into acetoacetyl-CoA. HMG-CoA synthase then combines acetoacetyl-CoA with another acetyl-CoA to form HMG-CoA, which is then cleaved by HMG-CoA lyase to regenerate an acetyl-CoA and produce the ketone body, acetoacetate. The majority of acetoacetate is reduced to βHB by βHB dehydrogenase. At this point, βHB, and some acetoacetate (in ~3:1 ratio), are exported into systemic circulation to be catabolized by the brain and other tissues [7] (Figure 1.2).

1.1.3 Ketone body oxidation

Ketone bodies, primarily βHB, pass the blood-brain barrier through monocarboxylate transporter (MCT) 1 and enter neurons or peripheral cells via this and other MCT isoforms. Once inside the mitochondria of target cells, βHB is oxidized back into acetoacetate by the reverse reaction of βHB dehydrogenase. The rate limiting step of ketone body oxidation is catalyzed by succinyl-CoA-3-oxaloacid CoA transferase (SCOT), which converts acetoacetate and succinyl-CoA into acetoacetyl-CoA and succinate. The latter, succinate, is a substrate for electron transport chain complex II, while the former, acetoacetyl-CoA, can be cleaved by ACAT into two acetyl-CoA molecules that can enter the Krebs cycle to generate NADH and $FADH_2$ and further support oxidative metabolism [7] (Figure 1.2).

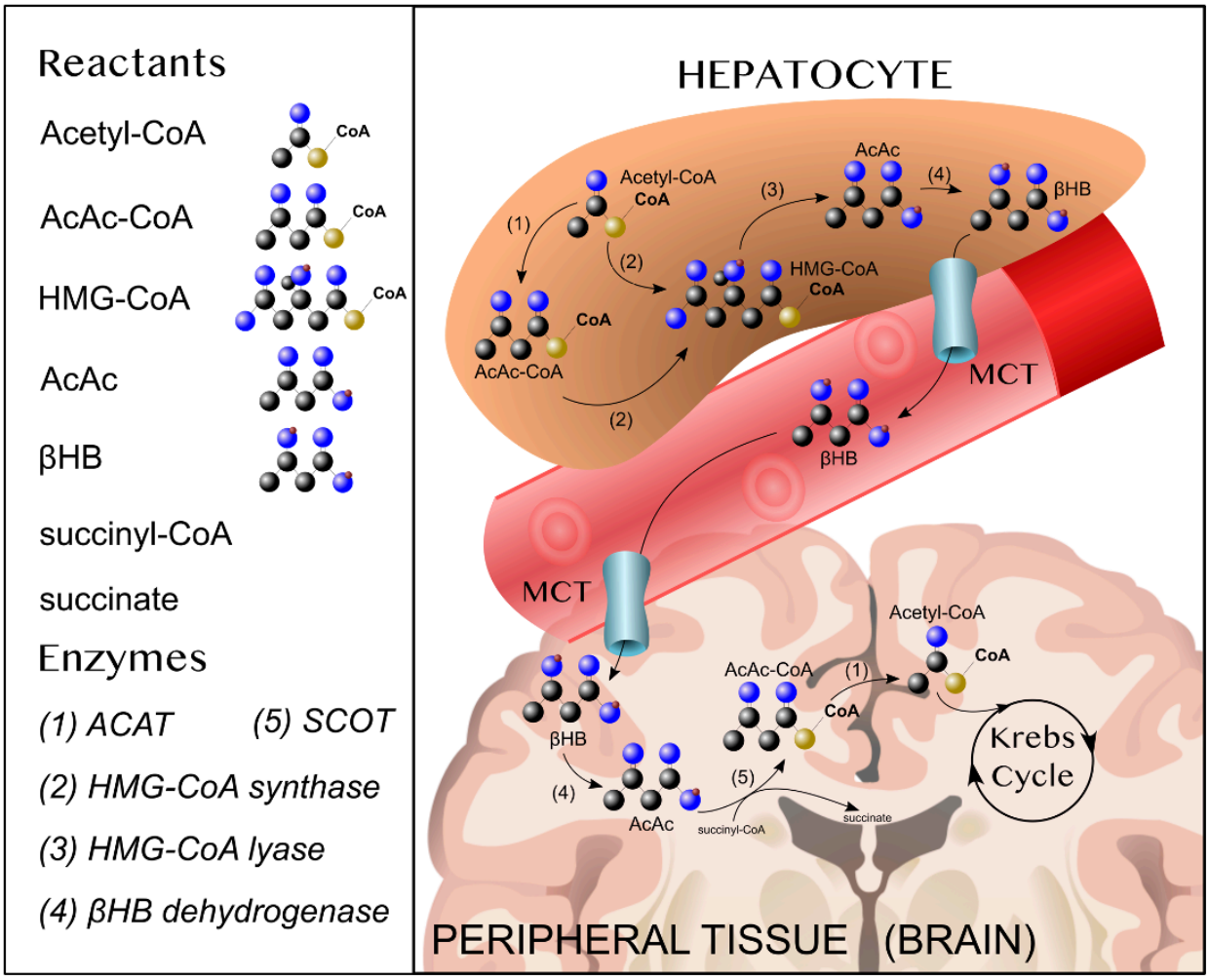


Figure 1.2 Endogenous ketogenesis and ketolysis

In hepatocytes, acetyl-CoA acetyltransferase (ACAT) (1) combines two acetyl-CoA molecules into acetoacetyl-CoA (AcAc-CoA). AcAc-CoA is combined with another acetyl-CoA by HMG-CoA synthase (2) to form 3-hydroxymethylglutaryl-CoA (HMG-CoA). HMG-CoA lyase (3) cleaves HMG-CoA, releasing acetyl-CoA and the ketone body, acetoacetate (AcAc). AcAc can then be reduced to βHB by βHB dehydrogenase (4). βHB, the main transport ketone, exits hepatocytes via monocarboxylate transporters (MCT) and travels through the circulation to peripheral tissues, including the brain. Once there, βHB is oxidized back into AcAc by βHB dehydrogenase (4). In the rate-limiting step of ketolysis, succinyl-CoA-3-oxaloacid CoA transferase (SCOT) (5) converts AcAc and succinyl-CoA into AcAc-CoA and succinate. AcAc-CoA is then cleaved by ACAT (1) to yield two molecules of acetyl-CoA that can enter the Krebs cycle. Black, blue, yellow, and red circles correspond to carbon, oxygen, sulfur, and hydrogen, respectively. (This figure and caption were adapted from Mota AS, Norwitz NG, and Clarke K. The original was composed by Norwitz NG.)

1.1.4 Exogenous ketogenic supplements

Ketone bodies are not found at meaningful levels in natural foods. Therefore, there has historically been no way for humans to ingest ketones bodies as a fuel source. This is a reason why, despite being a calorie-containing fuel substrate (in addition to carbohydrates, fat, and proteins), ketones are not popularly recognized as a fourth dietary macronutrient. However, the recent advent of exogenous ketogenic supplements has provided a means by which to induce ketosis without the need for fasting or carbohydrate restriction.

Different exogenous ketogenic substances exist. These include medium chain triglycerides, ketone salts, and ketone esters. Medium chain triglycerides, specifically those containing fatty acid chain lengths of six to ten carbons, are not true exogenous ketones because they require hepatic ketogenesis. They are ketogenic because, unlike long chain fatty acids, medium chain fatty acids are transported via the hepatic portal vein to the liver and do not require the carnitine shuttle for mitochondrial import. This means that medium chain triglycerides are converted into ketones faster than other fatty acids, earning them a reputation as an exogenous ketogenic substance.

Ketone salts and esters are two forms of true exogenous ketones. Ketone salts include ketone bodies, usually βHB, associated with salts, such as sodium. They are limited in their applicability in that they carry a salt burden, are often a racemic mix of natural D-βHB and its less bioactive enantiomer, L-βHB, and induce a relatively mild state of ketosis when compared to ketone esters. The ketone ester of relevance to this thesis is the monoester (R)-3-hydroxybutyl-(R)-3-hydroxybutyrate, heretofore referred to simply as “ketone ester” (KE). This KE is cleaved by gut esterases into βHB and butanediol, which are transported via the hepatic portal vein to the liver.

Once there, butanediol is converted by alcohol dehydrogenase to a second equivalent of βHB, such that each KE molecule yields two equivalents of pure D-βHB (Figure 1.3).

The advantages of KE are that it carries no salt load, is enantiomerically pure, and can induce a state of ketosis equivalent to that of multiple days of fasting within as little as thirty minutes. This makes it an ideal research tool for isolating ketosis as an independent variable, distinct from the multitudinous effects of starvation or carbohydrate restriction, as well as for studying the effects of ketosis in different disease states, including neurogenerative and neurological conditions.

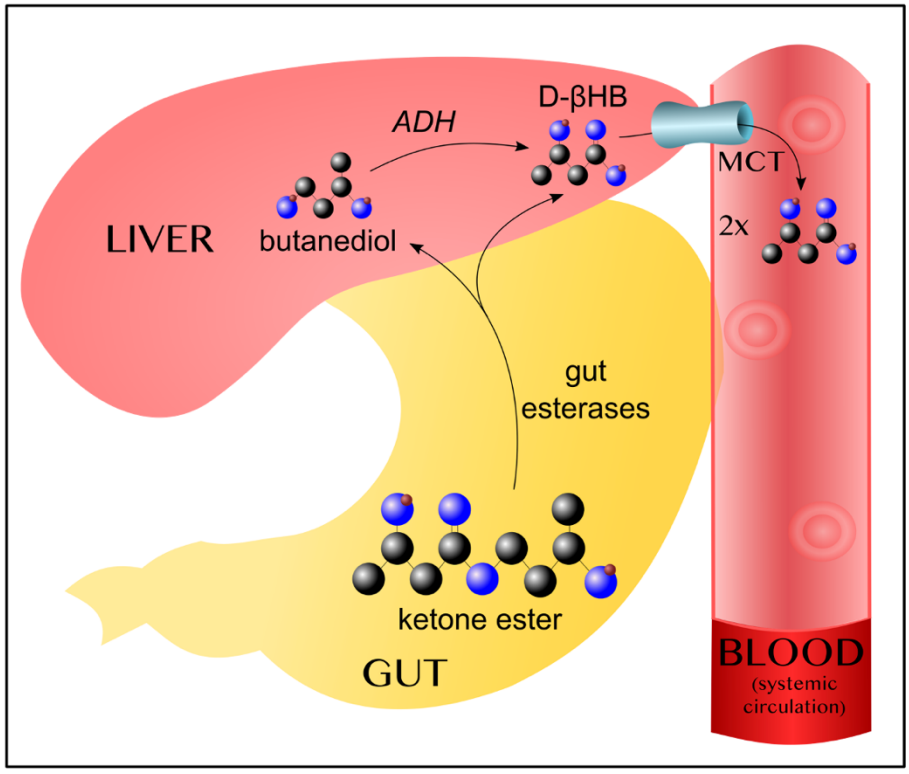


Figure 1.3 Digestion of ketone ester

The ketone ester bond is cleaved by gut esterases, yielding D-βHB and butanediol, which enter portal circulation. In the liver, alcohol dehydrogenase (ADH) converts butanediol into D-βHB, which leaves via monocarboxylate transporters (MCT). Each ketone ester molecule thus yields two D-βHB equivalents. Black, blue, yellow, and red circles correspond to carbon, oxygen, sulfur, and hydrogen, respectively. (This figure and caption were adapted from Mota AS, Norwitz NG, and Clarke K. The original was composed by Norwitz NG.)

1.2 Ketone ester for Parkinson's disease

1.2.1 Epidemiology

Parkinson's disease (PD) is the world's second most common and fastest growing neurodegenerative disorder. At present, PD affects 2–3% of individuals over the age of 65, a figure that is expected to double by the year 2040 [8, 9].

1.2.2 Pathological progression

In the brain, PD is characterized by the aggregation of misfolded α-synuclein protein. These α-synuclein aggregates do not build-up uniformly across the brain, but rather spread from region to region like a virus or, more accurately, a prion. In fact, it has been proposed that PD is a prion-like disease. According the Braak's staging hypothesis, α-synuclein starts in the olfactory bulb and the gut. While it remains relatively confined to the former, near the anterior of the cortex, it is thought to spread from the gut up the Vagus nerve to the brainstem, and then from there to the midbrain, a region that includes a group of cells called the substantia nigra (SN). The SN houses dopaminergic neurons that project to the striatum and influence the subcortical motor loop, which, in turn, communicates with the motor cortex and governs physical movements. The build-up of α-synuclein in the SN contributes to the death of SN dopaminergic neurons. Once 50 – 70% [10, 11] of SN neurons have died and dopamine is sufficiently depleted, the motor loop can no longer function, manifesting in the classical motor symptoms of PD.

It is important to note that the etiology of PD is monogenetic in only ~5-10% of known human cases [12]. Most cases are sporadic, triggered by upstream environmental and cellular pathologies that both induce α-synuclein to misfold and independently lead to the death of neurons in the SN

and other brain regions. Many different environmental triggers have been proposed, among the most notable of which, for the purposes of this thesis, is the pesticide, rotenone. Rotenone is a respiratory chain complex I inhibitor, exposure to which has been associated with PD [13]. Rotenone, along with the complex I inhibitor MPTP, are the most common means by which researchers generate animal and cellular models of PD.

While most human cases are probably not caused by pesticide exposure, complex I dysfunction and other energetic abnormalities are hallmarks of PD [14]. β-oxidation, the process by which fatty acids are broken down to generate $FADH_2$ and NADH for oxidative phosphorylation and acetyl-CoA for the Krebs cycle, is also impaired in PD [15]. These energetic abnormalities coincide with the pathologies of oxidative stress and inflammation. The former refers to an imbalance between the generation of reactive oxygen species (ROS) and cellular antioxidant defences. When an overabundance of ROS develops, these free radicals bounce around and damage membranes, proteins, and nucleic acids in a manner analogous to a bull in a cellular china shop. The latter, inflammation, refers to pathological overactivity of the immune system, which is analogous to the body's army. Chronic exposure to immune army activity causes collateral damage mediated by the weaponry of proinflammatory cytokine molecules. Chapters 7 (particularly sections 7.1 and 7.6) provides more details about the processes of energetic abnormalities, oxidative stress, inflammation, and cell death.

1.2.3 Neurodegenerative and neurological diseases as metabolic diseases

Energetic abnormalities (mitochondrial dysfunction), oxidative stress, and inflammation contribute not only to PD but also to all manner of neurodegenerative and neurological diseases and many other comorbid chronic diseases, such as cardiovascular disease and diabetes. These pathologies are what is being referred to throughout this thesis by the term, "metabolic diseases." Therefore, prior to returning to the discussion of PD specifically, it is important to highlight the three-step thread of logic that serves as the basis for this entire work:

(i) Neurodegenerative and neurological diseases, including PD, are metabolic diseases.

(ii) As metabolic diseases, they invite complementary metabolic approaches that address energetic abnormalities, oxidative stress, and inflammation.

(iii) Ketosis, induced either exogenously (KE) or endogenously (ketogenic diet), has potential to serve as such a metabolic approach.

1.2.4 Symptoms

The cardinal motor symptoms of PD include tremors, bradykinesia (slowness of movement), rigidity (stiffness), and postural instability (balancing issues). PD is also associated with a wide range of non-motor symptoms including loss of smell, gastrointestinal issues, disordered sleep, and cognitive dysfunction. When each symptom develops is highly variable among individual cases, but roughly corresponds to the spread of α-synuclein throughout the brain in the manner described above. Symptoms that often develop long before motor symptoms include loss of smell and gastrointestinal issues. Recall, according to the Braak staging hypothesis, α-synuclein begins in the olfactory bulb and gut. Disordered sleep may also occur before motor symptoms develop as the pathology affects the brainstem. After motor symptoms develop, some patients go on to develop symptoms associated with dysfunctions in the cortex, including cognitive decline and dementia.

The progression of PD is clinically defined by the Hoehn and Yahr scale, a 1 – 5 scale that classifies symptomology as follows:

1. Unilateral involvement with little-to-no functional disability
2. Bilateral involvement without impaired balance
3. Moderate disease with impaired postural reflexes but still physically independent
4. Severe disease but still able to stand
5. Confined to wheelchair or bed

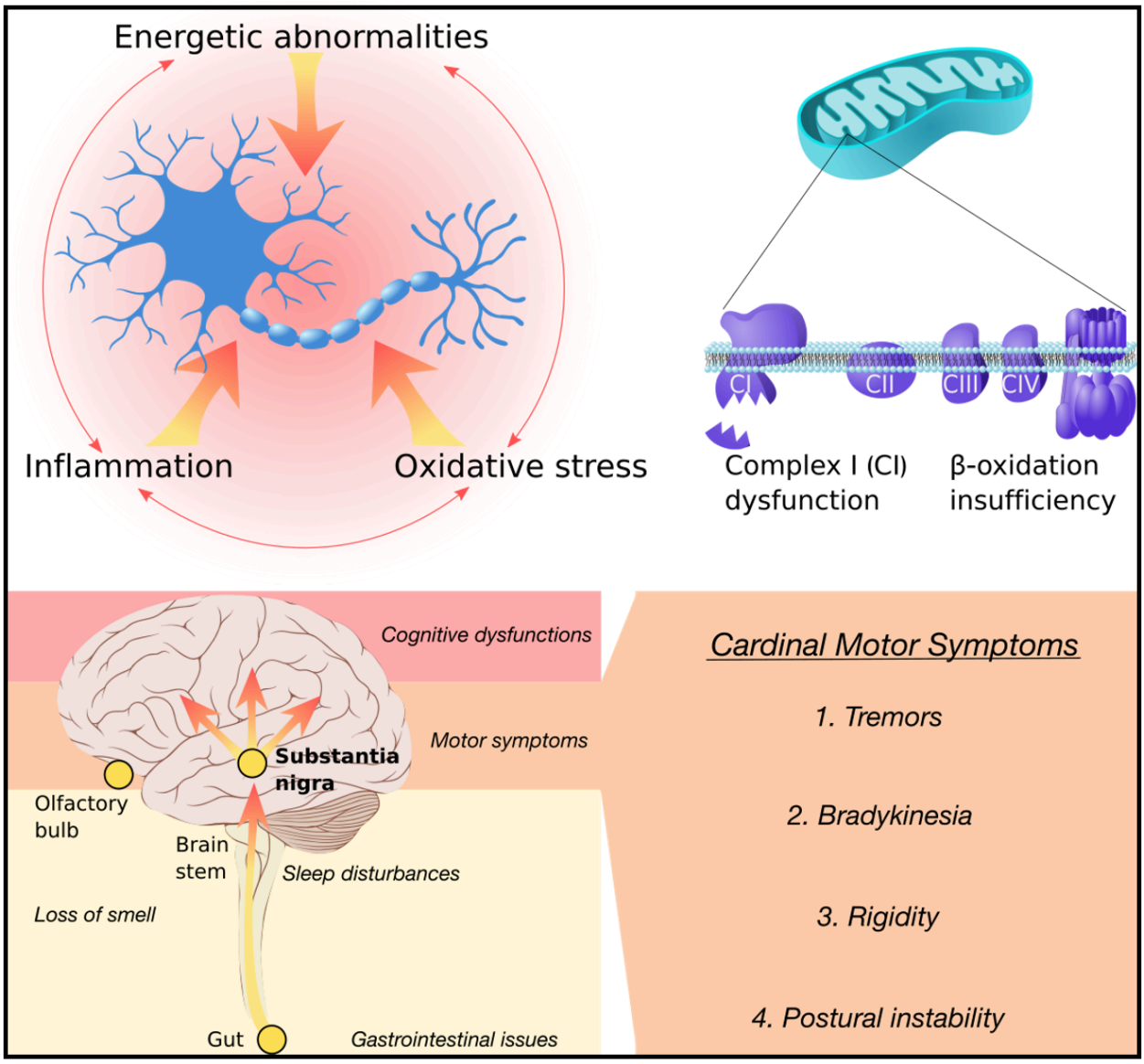


Figure 1.4 Pathologies and progression of Parkinson's disease

Parkinson's disease, as well as most other neurodegenerative and neurological diseases, are distinguished by the mutually reinforcing metabolic pathologies of energetic abnormalities (mitochondrial dysfunction), oxidative stress, and inflammation. Among the energetic abnormalities that distinguish Parkinson's disease are complex I dysfunction and β-oxidation insufficiency. Parkinson's disease is likewise distinguished by α-synuclein aggregates, the spread of which parallels the progression of symptoms. Early stage disease first affects the olfactory bulb and gut, progresses up the Vagus nerve to the brain stem, and often manifests in the coincident prodromal symptoms of loss of smell, gastrointestinal issues, and sleep disturbances. Only when the disease eliminates 50 – 70% of the dopaminergic neurons in the substantia nigra of the midbrain do the cardinal motor symptoms of Parkinson's disease – tremors, bradykinesia, rigidity, and postural instability – develop. Later disease may involve not only the progression of motor symptoms, but also affect the cerebral cortex and manifest as cognitive dysfunctions and possibly in dementia.

1.2.5 Selective vulnerability of substantia nigra dopaminergic neurons

If PD is a "metabolic disease," as stated above, the following question arises: why is PD characterized by the relatively specific death of SN dopaminergic neurons?

1.2.5a Extensive, unmyelinated axonal arborization

The selective vulnerability of SN dopaminergic neurons may, in part, be due to the energetic demands imposed by their axonal morphology. In rats, a single SN dopaminergic neuron possesses an axonal arbor that totals around sixty centimeters in length and gives rise to approximately one quarter million striatal synapses, a figure that is orders of magnitude larger than the number of synapses formed by other neurons in the rat brain. Extrapolating to humans, based on the relative volume of striatum and number of SN dopaminergic neurons, a single human SN dopaminergic neuron may possess an arborization pattern that totals 4.5 meter in length and makes as many as 250 million synapses in the striatum [16].

In support of the supposition that the SN dopaminergic neurons' extensive axonal arbor contributes to their selective death in PD, dopaminergic neurons of the ventral tegmental area are relatively spared in PD [17, 18]. Correspondingly, this latter population of dopaminergic neurons is characterized by smaller axonal trees that make only about one-tenth the synapses of dopaminergic neurons in the SN [19].

In addition to their pure gigantism, SN dopaminergic neuron axons are unmyelinated. This further increases energetic demand because myelin sheaths insulate axons to allow for action potential propagation via saltatory conduction. The absence of myelin insulation increases the need for, and

density of, sodium channels along the axon and increases the energetic cost of propagating action potentials and maintaining membrane potential [20].

The energetic demands of sustaining this extensive, unmyelinated axonal arbor – including protein synthesis, cytoskeleton maintenance, axonal transport, synaptic transmission, and propagating action potentials – puts SN dopaminergic neurons, in the words of Bolam and Pissadaki, energetically "on the edge" [16]. In brief, the energy-demanding nature of this axonal arbor makes SN dopaminergic neurons particularly susceptible energetic stressors and suggests that this neuron population would be disproportionately affected by metabolic dysfunctions.

1.2.5b Intrinsic calcium pacemaking

SN dopaminergic neurons are also characterized by calcium pacemaking activity mediated by $Ca_v1.3$-containing L-type calcium channels. This intrinsic calcium pacemaking activity is required to maintain basal dopaminergic tone and functional physical movement; however, it is extremely metabolically expensive. The relative intracellular and extracellular concentrations of sodium, potassium, and calcium afford a degree of insight. Whereas the transmembrane gradient of sodium and potassium are maintained at 10-30-fold differences across the membrane, the concentration of calcium is roughly 20,000-fold higher outside neurons as compared to inside. As the energy required to move an ion up its electrochemical gradient increases as the steepness of the gradient increases, the energetic expensiveness of maintaining the transmembrane potential of calcium ends up being on the order of eight greater than that of sodium [21].

Evidence that L-type calcium channels and SN dopaminergic neurons' pacemaking activity contributes to their selective vulnerability in PD can be derived from comparisons to other neuronal groups. As stated above, ventral tegmental area dopaminergic neurons are spared in PD, at least as compared to those in the SN. Not only do the former group of dopaminergic neurons possess smaller axons that make fewer synapses, but they do not manifest the same calcium oscillations and have a lower density of L-type calcium channels as compared to SN dopaminergic neurons [22]. Additionally, other non-dopaminergic cell groups that are at risk in PD – such as cells in the dorsal motor nucleus of the vagus (cholinergic), locus ceruleus (noradrenergic), and raphe nuclei (serotonergic) – likewise possess energetically expensive intrinsic calcium pacemaking activity. And, all these cell groups are further characterized by broad calcium spikes, permitting more time for calcium entry and, thus, imposing a larger energetic burden [21].

1.2.5c Systemic rotenone administration model

Administration of MPTP or rotenone are the most common means by which to develop animal models of PD. Both of these toxins are complex I inhibitors, which is appropriate given that systemic complex I dysfunction is a pathological feature of PD. Complex I is impaired in patients with PD, not only in the brain [23, 24], but also in platelets [25], muscle cells [26], and peripheral blood mononuclear cells [27]. And, yet, in both idiopathic human PD and MPTP or rotenone animal models, SN dopaminergic neurons die en masse, while most other neurons are spared. Why?

Whereas the selectivity of MPTP for SN dopaminergic neurons derives partially from the fact that its metabolite, MPP^+, is a substrate for dopamine transporters, rotenone is hydrophobic and can

cause uniform complex I inhibition across the brain. Still systemic administration of rotenone causes the specific death of SN dopaminergic neurons. For example, Sherer and colleagues demonstrated that chronic systemic administration of 2.0 – 3.0 mg/kg/day rotenone to rats recapitulated cardinal motor symptoms of PD, in conjunction with aggregation of α-synuclein (possibly due to oxidative damage and/or decrease proteasome or autophagy-mediated degradation downstream of mitochondrial dysfunction) and selective death of SN dopaminergic neurons. Other dopaminergic neurons, such as those in the nucleus accumbens and ventral tegmental area, were spared and no remarkable degeneration was observed in any other basal ganglia nuclei [28]. The rotenone model has since been demonstrated to be highly reproducible [29].

The systemic rotenone model dovetails with the hypothesis put forth by Bolam and Pissadaki, above, that SN dopaminergic neurons are selectively vulnerable to metabolic dysfunctions because they are energetically "on the edge" [16]. Otherwise stated, SN dopaminergic neurons are more susceptible to bioenergetic stress because they have a smaller "respiratory reserve" – the difference between their basal ATP requirements and capacity to produce ATP by oxidative phosphorylation [21] – and, thus, more likely to be pushed "over the edge" by energetic stressors, be those genetic or environmental.

1.2.6 Absence of disease-modifying treatment

The standard of care for PD is levodopa, which helps compensate for dopamine deficiency by simply providing the brain with a dopamine precursor. However, such dopamine replacement therapy only provides a symptomatic bandage. Dopaminergic neurons continue to die and the underlying disease progresses.

1.2.7 Rationale for ketone ester

βHB is both a fuel substrate for failing neurons, that may have impaired glucose metabolism, and also a potent signaling molecule. By correcting or compensating for energetic abnormalities, reducing oxidative stress and inflammation, and protecting against cell death, βHB (and by extension KE) has the potential to address the symptoms of PD and also address the underlying pathologies of PD [30]. The mechanisms and disease-modifying potential of βHB are the topic of chapter 7, in particular section 7.1.

1.2.8 Study aims

The aims upon which this thesis was originally based correspond to the three clinical studies presented in chapters 3 – 5 and are as follows:

Chapter 3: Vigorous exercise has been hypothesized to be among the only disease-modifying strategies for PD. It addresses the aforementioned metabolic pathologies, and there is evidence that it decreases the risk of developing disease and that it slows disease progression [31-34]. Correspondingly, KE has been shown to improve exercise performance in elite athletes [35].

- *Aim 1 was to investigate whether a single dose of KE could improve endurance exercise performance in PD, potentially providing a way to enhance the efficacy of exercise therapy.*

Chapter 4: PD is associated with a decline in ATP levels (as part of energetic abnormalities) in the SN and other afflicted brain regions [36]. This energy crisis in neurons is thought to contribute to cell death and disease progression.

- *Aim 2 was to test whether a single dose of KE could increase ATP levels in the brains of patients with PD.*

Chapter 5: PD is associated with a wide array of both motor and non-motor symptoms (any of which could be affected by KE), and ketogenic diets have been shown to positively impact PD symptomology over the course of one month [37, 38].

- *Aim 3 was to investigate whether KE supplementation, given four times daily over 28-days, might impact any PD symptoms.*

1.3 Broadening of scope

When the COVID-19 pandemic forced the suspension of the third study, it was determined that the study would be included as a validation of methods and feasibility study and that the absent data would be supplemented with additional contributions made to the fields of ketogenics and neurodegeneration and neurological diseases.

The first of these additional contributions is an original nutrition tracking application for research and clinical use named, “My Ultimate Nutrition Calculator and Helper” (MUNCH). MUNCH has the potential to enable future nutrition research studies – including and especially those on low-carbohydrate and ketogenic diets – that collect accurate data over long periods of time, and to do so in an inexpensive manner.

Beyond the original contributions of the three clinical studies and MUNCH application, this thesis includes eight further peer-reviewed manuscripts – reviews, theory papers, and one case report – that collectively offer novel and nuanced perspectives on ketogenics and neurodegenerative and neurological diseases.

Chapters 6 – 8: The MUNCH application and published manuscripts represent a fourth supplemental aim of this thesis:

- *Supplemental Aim 4 was to demonstrate continued productivity, intellectual competence, and scientific writing capability under the circumstances of the COVID-19 pandemic.*

Chapter 2. *General methods*

2.1 Ethics

2.1.1 ISRCTN registration

All three Parkinson's disease (PD) clinical studies were first registered on the international standard randomized controlled trial serial number (ISRCTN) database, a registry for clinical trials that promotes transparency in human research and supports unbiased reporting. The ISRCTN is recognized by the World Health Organization and the International Committee of Medical Journal Editors as an appropriate repository for planned, in process, and complete clinical trial parameters.

Every effort was made to keep the registry updated with respect to recruitment status, overall trial status, and study parameters. The ISRCTN codes for the three clinical studies are as follows:

(i) Endurance exercise study (chapter 3): ISRCTN16599164

(ii) ^{31}P-MRS study (chapter 4): ISRCTN10531043

(iii) 28-day study (chapter 5): ISRCTN64294760

2.1.2 REC and HRA approvals

Following study registrations, all clinical studies associated with the National Health Service (NHS) require approvals by independent Research Ethics Committee (REC) and Health Research Authority (HRA) boards.

Approvals were sought through the Integrated Research Application System (IRAS), which is a multistep process that requires forms covering the following of matters: administrative details, research overview, research purpose and design, risks and ethical issues, possible benefits, protocols and procedures, recruitment information, confidentiality information, publication and dissemination statements, scientific and statistical review, management forms, details of products and devices, and research site reviews.

Following a complete application and initial HRA validation, a REC was assigned and the procedures and protocols put forth in the application were defended at in-person meetings. After any and all REC board member concerns were addressed, the REC committees issued approvals and the applications were sent on for HRA assessment and received HRA approvals.

The IRAS project application identifiers, REC committee names, and REC reference codes for each study are as follows:

(i) Endurance exercise study (chapter 3)

IRAS project ID: 257795

South Central - Oxford A Research Ethics Committee

REC reference: 19/SC/0032

(ii) ^{31}P-MRS study (chapter 4)

IRAS project ID: 257793

South Central - Oxford A Research Ethics Committee

REC reference: 19/SC/0033

(iii) 28-day study (chapter 5)

IRAS project ID: 256914

South Central - Oxford B Research Ethics Committee

REC reference: 19/SC/0138

Samples of REC and HRA approvals are given in Appendix 2.

2.2 Contracts

The ketone ester (KE) in PD clinical studies constituted an academic project supported by an industry sponsor (TDeltaS Ltd., founded by the University of Oxford's Professor Kieran Clarke) being conducted within the NHS system at the John Radcliffe hospital with vulnerable participants. Therefore, a number of contracts to coordinate the associated institutions were needed. The institutions included the University of Oxford, Oxford Brookes University, TDeltaS Ltd., the John Radcliffe hospital, and other bodies within the NHS.

The functions of each contract are briefly summarized in sections 2.2.1 - 2.2.9.

2.2.1 Collaboration agreements

These agreements clarified the financial obligations, supply of materials, intellectual property rights, and privilege to and protection of data of the aforementioned bodies (University of Oxford, Oxford Brookes University, TDeltaS Ltd., and the John Radcliffe hospital).

2.2.2 Trust management approval

The research and development offices of Oxford University Hospitals NHS Foundation Trust need to confirm the capacity and capability of clinical studies conducted in the NHS system. The trust management agreements required evaluations of study feasibility with respect to finances, patient safety, and use of hospital space.

2.2.3 Confidentially agreements

As these studies were being supported by a commercial sponsor (TDeltaS Ltd.), agreements between the National Institute for Health Research and the sponsor were required that clarified how confidential patient and hospital information passed onto the sponsor could and could not be used. The restrictions set out in these agreements protected privileged information and patient confidentiality.

2.2.4 Insurance

Certificates and confirmation of insurance from the study sponsor, TDeltaS Ltd. (CFC Underwritings policy number: BSH03627830) provided evidence that unforeseen financial liabilities, as in those occurring due to unexpected adverse events, would be covered up to a cost of five million pound sterling.

2.2.5 Participant identification center

As patients were being recruited out of the OPDC for the endurance exercise study taking place at a non-NHS site, permissions were required to have the John Radcliffe hospital serve as a participant identification center for Oxford Brookes University.

2.2.6 Test permissions

Certain tests and questionnaires included in the clinical studies are under copyright and require permissions to employ in new research. Examples include the Movement Disorder Society Unified Parkinson's Disease Rating Scale (MDS-UPDRS) and Montreal Cognitive Assessment (MoCA)

used in the 28-day study. Appropriate permissions were obtained from the relevant parties for each test included in the protocols.

2.2.7 Test costings

The budget and study costing forms required by the NHS and study sponsor necessitated quotes for the costings of both on-site and off-site test materials. These included precise expense report forms or quotes for materials such as blood strips and tubes, clinic room time, lab analyses, and transport fees.

2.2.8 Services agreements

Specific service and licensing agreements were required by Oxford University Hospitals NHS Foundation Trust to clarify how facilities at the John Radcliffe hospital would be used. Specifically, the services agreements explained how supplies would be provided, how samples would be handled, what technical support would be required, how confidential information would be handled, who would have the rights to intellectual property, and when specific services would be required.

2.2.9 NHS worker and Brookes student contracts

Separate worker contracts are required for student researchers to operate within the John Radcliffe Hospital and Oxford Brookes University. The contract to the former required off-site interviews, and that to the latter required an independent application to Oxford Brookes in order to be enrolled as a student before a work contract could be sought.

2.3 Participant criteria

2.3.1 Inclusion criteria

To be included in any of the studies, participants were screened for the following criteria:

(i) Diagnosis of PD

(ii) Taking levodopa

(iii) Hoehn and Yahr stages 1 – 2

(iv) Fluent in English

(v) Capable of giving informed consent

2.3.2 Exclusion criteria

Participants were excluded from the studies if they fulfilled any of the following criteria:

(i) Communication impairments

(ii) History of cardiovascular disease

(iii) Failed electrocardiogram

(iv) Contraindications for undergoing magnetic resonance imaging

(v) Any other significant disease or disorder which put the participants at risk because of participation in the study or could influence the results of the experiments

Criteria (ii) - (iii) were specific to the endurance exercise study and criteria (iv) was specific to the ^{31}P-MRS study.

2.4 Recruitment

2.4.1 Recruitment pipeline

Participants were recruited from the Oxford Parkinson's Disease Centre (OPDC) cohort. To maintain patient confidentiality, initial contact could only be made by OPDC team members. The John Radcliffe hospital, base of the OPDC, was therefore chosen as participant identification center for all three studies. After participants granted permission for further contact, participants were sent information sheets corresponding to the study of interest, a sample of which is given in Appendix 3.

2.4.2 Informed consent

After affording participants time to review the information sheets, participants were called on the phone to discuss their questions and concerns and to verbally clarify the exact nature of the study, including what it would involve from the participant, the implications and constraints of the protocol, the confidentiality of personal data, and the known side effects and risks involved in taking part. At their first on site visits, participants were again asked to read the information sheets immediately prior to signing informed consents, a sample of which is given in Appendix 4.

2.5 Standard experimental procedures

2.5.1 Trainings

Prior to commencing the studies, certain trainings and certification programs deemed to be necessary or helpful were completed. These included the following:

(i) Phlebotomy (Maxis Healthcare)

(ii) Cardiopulmonary resuscitation (St John Ambulance)

(iii) Automated external defibrillation (St John Ambulance)

(iv) Good clinical practices (National Health Institute for Research)

(v) Information security (University of Oxford)

(vi) Research integrity (University of Oxford)

(vii) Magnet safety (Oxford Center for Clinical and Magnetic Resonance)

2.5.2 Sample size selection

Target sample sizes were determined for each study given a power of 0.80 and an alpha level of 0.05 (two-sided). First, the primary outcome metrics upon which the sample size analyses would be based was chosen. Then minimum meaningful effect sizes and variance values for these primary outcome variables of interest were identified based on previous literature.

The specific formula used for such calculations is shown below, where Z_α and Z_β correspond to z-statistics on a normal distribution for the set alpha and beta values (when alpha = 0.05, on a two-sided test, $Z_\alpha = 1.96$; when beta = 0.80, on a two-sided test, $Z_\beta = 0.84$), σ is the variance, and Δ is the effect size.

$$n = \frac{2\ x\ \left(Z_\alpha + Z_\beta\right)\ x\ \sigma^2}{\Delta^2}$$

In simple terms, what one might consider a meaningful effect on the primary outcome variable, and what natural variance one might expect in the primary outcome variable, were derived from the literature. Given this meaningful effect and variance, as well as a standard p-value cutoff of 0.05, it was calculated how many participants would be needed such that the study had an 80% chance of correctly rejecting the null hypothesis.

2.5.3 Randomization

Participants were randomized using a block-four protocol in which every four recruited participants were segregated equally into the KE and placebo arms. A random number generator was used to assign study specific number codes to participants. Odd numbers were segregated to the KE group and even numbers were segregated to the placebo group for the first two participants. If the first two participants were allocated to separate groups, the same rule then applied to the third participant. The fourth participant was placed in the group with the remaining space.

2.5.4 Fasting and medications

The participant information sheets instructed participants to come to the laboratory before breakfast and before having taken any medications. The evening before each visit, participants were called to remind them not to consume any food or drink, other than water, or to have any medications the morning before the tests. The morning of each visit, participants confirmed that they were fasted, had not taken medications, and had not had any drink other than water.

2.5.5 Blood handling and analysis

Participants' blood glucose and βHB were measured using a fingerstick assay and handheld FreeStyle Precision Neo Pro monitor. The pad of the middle or index finger was sanitized with an ethanol swab prior to drawing blood half of an inch from the center of the pad. The first drop of blood was discarded prior to measuring glucose and βHB. The monitor readouts were shielded such that participants could not infer, from the presence or absence of ketosis, whether they were in the KE or placebo group.

2.5.6 REDCap data storage and sharing

The John Radcliffe Hospital and OPDC use the Research Electronic Data Capture (REDCap) system for the electronic organization of study events as well as the storage of study data. This system was therefore used to store study data in a manner that was both compliant with the Data 2018 Protection Act and enabled verified individuals within the research team, such as the chief investigator, to have easy access the data.

2.5.7 Procedural amendments

As the studies commenced, modifications to the protocols that would either improve data quality or reduce participant burden were made. This is a normal in the conduct of clinical studies and is encouraged as certain procedural modifications only become possible after the initial ethical approvals were issued.

For example, it was determined that the My Ultimate Nutrition Calculator and Helper (MUNCH) tool could be implemented in a subset of individuals in the 28-day study to investigate the impact of specific dietary components and macronutrient ratios on participants' degree of ketosis and responsivity on tests. It was not possible to include this proposal in the initial application as MUNCH, which is the topic of chapter 8, was not in development at the time.

Amendments to protocols that impact data output or alter participants' responsibilities, as above, are classified as "substantial" by the HRA and require evaluation by REC and HRA boards. All amendments received approvals before the protocols were updated.

2.6 Confidentiality and safety

2.6.1 Data privacy

Participant anonymity was maintained by only using unique participant identification numbers on all study documents and on the REDCap system. The only exceptions were the enrolment log and written informed consents.

In accordance with the 2018 Data Protection Act, data were anonymized as soon as practical. All documents were secured on-site at the John Radcliffe Hospital and Oxford Brookes in locked cabinets only accessible by study staff and authorized personnel.

2.6.2 Adverse event reporting

In order to support patient safety, it was important to establish guidelines for reporting any adverse events that could occur during the studies, whether they resulted from the studies' procedures or were merely coincident with them. The steps established for recording adverse events included recording the description, date of onset, and end date for the event, as well as having a medically qualified member of the research team evaluate the likelihood that the event was or was not due to an intervention associated with the study and evaluate the severity of the event based on a numerical scale: 1 = mild, 2 = moderate, 3 = severe. The protocol and ethical approvals required that all severe events be reported to the sponsor within 24 hours of their onset and to the corresponding REC within one week. Fortunately, no such severe events occurred for the endurance exercise study nor the ^{31}P-MRS study. These adverse event reporting rules stand for the ongoing 28-day study.

2.7 List of general study documents

Ethics & Amendments	Contracts	General Study Documents
IRAS application forms	Collaboration agreements	Protocols
REC approval letters	Trust management approvals	Participant information sheets
HRA approval letters	Confidentiality agreements	Consent forms
Substantial amendment approvals	Certificates of insurance	General practitioner letters
	Participant identification centers	Statements of activities
	Test permissions	Schedules of events
	Test costings	Responsibility logs
	Service and licensing agreements	Questionnaires and surveys
	Honorary Worker Contracts	Adverse events forms
		Complete study site file

Table 2.1 List of general study documents

This table summarizes the documentation required for the setup of the three clinical studies, with each item being required for each study. It does not constitute an exhaustive list of the documentation required to obtain particular contracts or approvals, but only the end results.

Chapter 3. *A ketone ester drink enhances endurance exercise performance in Parkinson's disease*

Declaration

I certify that the contents of this thesis chapter, and the associated manuscript, represent my own ideas, work, and words.

Norwitz NG, David J. Dearlove, Meng Lu, Kieran Clarke, Helen Dawes, Michele T. Hu. A ketone ester drink enhances endurance exercise performance in Parkinson's disease. *Frontiers in Neuroscience*. 2020

3.1 Abstract

Objectives: Routine exercise is thought to be among the only disease-modifying treatments for Parkinson's disease; however, patients' progressive loss of physical ability limits its application. Therefore, we sought to investigate whether a ketone ester drink, which has previously been shown to enhance endurance exercise performance in elite athletes, could also improve performance in persons with Parkinson's disease.

Participants: 14 patients, aged 40-80 years, with Hoehn and Yahr stage 1-2 Parkinson's disease.

Intervention: A randomized, placebo-controlled, crossover study in which each participant was administered a ketone ester drink or an isocaloric carbohydrate-based control drink on separate occasions prior to engaging in a steady state cycling test at 80 rpm to assess endurance exercise performance.

Outcomes Measures: The primary outcome variable was length of time participants could sustain a therapeutic 80 rpm cadence. Secondary, metabolic outcomes measures included cardiorespiratory parameters as well as serum β-hydroxybutyrate, glucose, and lactate.

Results: The ketone ester increased the time that participants were able to sustain an 80 rpm cycling cadence by 24% ± 9% ($p = 0.027$). Correspondingly, the ketone ester increased β-hydroxybutyrate levels to >3 mmol/L and decreased respiratory exchange ratio, consistent with a shift away from carbohydrate-dependent metabolism.

Conclusion: Ketone ester supplementation improved endurance exercise performance in persons with Parkinson's disease and may, therefore, be useful as an adjunctive therapy to enhance the effectiveness of exercise treatment for Parkinson's disease.

3.2 Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the world, affecting 1% of adults over the age of 60 and growing in prevalence at an alarming rate [39, 40]. PD is identified pathologically by the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain, which disrupts subcortical motor loop function and leads to the classic motor symptoms of bradykinesia, tremor, rigidity, and postural instability. There are currently no disease modifying drug therapies for PD. Standard treatment involves dopamine replacement with levodopa, a medication that has a temporary window of efficacy, can induce dyskinesias, and does not address the burdensome nonmotor symptoms of PD [41].

By contrast, moderate to vigorous exercise is thought to be among the only treatments may change the disease's course. Certainly, it is near impossible to prove that exercise is disease modifying in PD, as to do so would require large long-term prospective trials in which patients were randomized to exercise therapy or a control. However, the combination of animal model data showing the vigorous exercise is neuroprotective, and retrospective epidemiological studies showing moderate to vigorous exercise is associated with reduced risk, together support the notion that exercise might be a disease-modifying treatment for PD.

Animal models of PD have demonstrated that physical activity is neuroprotective against dopaminergic toxins, such as 6-OHDA and MPTP. For example, rats forced to routinely run on a treadmill shortly after being injected with 6-OHDA exhibited preserved motor function and retained twice as many SNpc dopaminergic neurons as non-exercised controls [42]. Exercise dosage also appears to be important. In an MPTP murine model of PD, three months of exercise

prior to SNpc lesion protected against dopaminergic neuron and striatal dopamine losses, but not when the exercise was restricted to two-thirds or fewer wheel revolutions [43]. These and other models demonstrate that exercise attenuates motor symptom deficits, in association with the preservation of dopaminergic neurons and/or dopaminergic neurotransmission [44].

The animal literature dovetails with retrospective studies demonstrating vigorous exercise in midlife reduces the risk of developing PD [31-33]. In the largest of these studies, exercise history, including both light and moderate to vigorous exercise, was assessed in a population of over 200,000 participants. After adjusting for potentially confounding variables, the study found that moderate to vigorous exercise at midlife was associated with 38% reduced risk of developing PD. However, no significant reduction in risk was observed for light physical activity. This study also included a meta-analysis of the existing retrospective trials examining the relationship between past physical activity and the development of PD, which all report reductions in disease risk of similar magnitude (overall 33% reduction in risk) [31]).

Recently, a phase II randomized clinical trial, the Study in Parkinson's Disease of Exercise (SPARX), reported that high-intensity treadmill exercise, but not moderate intensity exercise, prevented progression of motor symptoms over six months in newly diagnosed patients. SPARX included 128 participants with recently diagnosed PD who were randomized into high-intensity, moderate-intensity, and sedentary control groups. After six months, the high-intensity exercise group exhibited no change in UPDRS motor score, whereas both the moderate intensity and control groups exhibited declines in motor function [45]. The animal data, retrospective epidemiological studies, and SPARX trial all suggest that vigorous exercise is disease-modifying in PD.

How vigorous physical exercise exerts its disease-modifying potential in persons with PD disease is unknown. However, a range of possible mechanisms exist and include exercise-induced increases in the expression of neurotrophic factors, like GDNF and BDNF [46, 47], which are depleted in the PD brain [48]; enhancement of dopaminergic neurotransmission via increased vesicular release of dopamine as well as the restoration of dopamine receptors [49, 50]; increased VEGF and regional blood flow [51]; or improvements in oxidative stress, inflammation, and immune signaling [50, 52]. It is likely that any clinical effect would be a combination of these and other undiscovered mechanisms.

Our study used cycling as a mode of exercise. Prior interventional studies examining the therapeutic effects of cycling exercise in PD have emphasized cadence as the critical variable. Patients who cycle at higher cadences of around 80 revolutions per minute (rpm) exhibited motor benefits that persist for at least a month and are not observed in those who exercise at lower cadences [53, 54]. For the purposes of this study, we took 80 rpm to be a therapeutic threshold. Unfortunately, the possibility that 80 rpm cycling confers benefits in PD presents a paradox. The progressive nature of the motor disease undermines the long-term efficacy of vigorous exercise as a potentially neuroprotective strategy. The purpose of this study was to explore whether a nutritional supplement could boost exercise performance in PD, thereby better enabling patients to engage in what may be the only available disease-modifying treatment for their condition.

Specifically, we chose to examine whether a ketone ester (KE) supplement, ΔG®, could increase patients' ability to sustain an 80 rpm cycling cadence. KE provides the bioidentical ketone molecule, D-β-hydroxybutyrate (βHB), to that produced by the human body during periods of

carbohydrate scarcity to fuel the human brain and muscles [30]. Furthermore, it is proven safe [55], improved cycling performance in elite athletes [35], and, particularly in PD, may help to circumvent the respiratory chain complex I blockade and recover intramuscular β-oxidation [15, 30, 56]. In the discussion, we elaborate up these and other mechanisms of action by which KE could improve exercise performance in PD and explain why the βHB molecule, which itself has neuroprotective properties in PD, adds to the promise that needed disease modifying treatments for PD are on their way.

3.3 Methods

3.3.1 Participants and screening

This study (registered ISRCTN16599164) was approved by external research ethics committees (National Health Service Health Research Authority and South-Central Oxford A Research Ethics Committee, REC reference: 19/SC/0032) and was conducted in accordance with the declaration of Helsinki (2008). Fifteen participants were recruited from the Oxford Parkinson's Disease Centre (OPDC) Discovery cohort and the study was conducted at Oxford Brookes University, Headington campus, between July and December 2019. Participants were Hoehn and Yahr stages 1 - 2, between the ages of 40 and 80, non-smokers, and did not possess a condition, other than PD, that would restrict their ability to exercise. Levodopa equivalent daily dose (LEDD) was calculated using methods previously reported [57]. Participants provided written informed consent prior to inclusion. Following informed consent, but prior to exercise, participants underwent a resting twelve-lead electrocardiogram. One participant was dismissed because of an irregular cardiac rhythm, bringing the total number of recruited and completed participants to n = 14. Participant characteristics are shown in Table 3.1.

Age (years)	62.2 ± 1.5	Male / Female	10 / 4
Height (meters)	1.70 ± 0.02	Disease duration (years)	4.9 ±1.0
Weight (kg)	72.4 ± 3.3	Hoehn and Yahr	1 - 2
BMI (kg/m^2)	25.1 ± 1.1	Levodopa equivalent dose	520 ± 93

Table 3.1 Participant characteristics

Values are reported as means ± standard error.

3.3.2 Baseline test

To determine the appropriate fixed wattage at which to set the cycle ergometer for subsequent testing, participants engaged in a baseline test. This test also served as a familiarization session for subjects, allowing them to become accustomed to the laboratory and exercise equipment before the 80 rpm endurance test (below). Participants came into the lab at either 8:00 or 10:00 am fasted (> 8 hours) and following overnight withdrawal of dopaminergic therapy (levodopa and dopamine agonists), termed "off" medication. After being cleared for exercise by a resting twelve-lead electrocardiogram and Physical Activity Readiness Questionnaire, participants were instructed to cycle 80 rpm for as long as possible on an electronically braked cycle ergometer (Excalibur Sport, Lode Netherlands), integrated with a cardiopulmonary monitoring system (Metalyzer 3B, Cortex, Germany) that controlled the work rate protocol on the ergometer. The test began with a 4-minute warmup set at 50 Watts. Thereafter, wattage increased by 10 Watts every 2 minutes until the participant stated s/he was too tired to continue or could not sustain a cadence of >70 Watts for a cumulative total of 20 seconds. Resting heart rates, heart rates taken at the end of each step, and approximated maximum heart rate (220 - age) were used to create a linear regression of wattage as a function of heart rate. Each participant's fixed-Watt value for subsequent tests was set at 55% of their projected wattage at maximum heart rate. In order to standardize test length, some

participants' warmup was set to 25 Watts and some participant's fixed-Watt value was set at 65% of their projected wattage at maximum heart rate. These adjustments helped to compensate for individual participants' exercise tolerances for subsequent endurance testing. Absolute load did not impact outcomes and the randomized cross-over design ensured that these necessary personalized adjustments on the baseline test did not impact the integrity of the data.

3.3.3 Drink preparation and randomization

The two drink preparations used in this study were a ketone ester plus carbohydrate (KE+CHO) drink, consisting of 25 mL ΔG® and 45 g dextrose diluted in an additional 25 mL water, and an isocaloric (300kCal) taste-matched carbohydrate (CHO) drink, consisting of 75 g dextrose and 1.5 mL Symrise bitter flavor (product code: SY648352) dissolved in 50 mL water. Drinks were prepared in a room separate from participants and administered in an opaque container. Their viscosity and taste profiles were matched such that participants could not discriminate between the drinks.

As this was a randomized crossover study, each of the fourteen participants took part in both arms of the study on testing days that were separated by one week. The order in which the KE+CHO and CHO drinks were presented was determined by a random number generator, with even numbered participants receiving KE+CHO first and odd numbered participants receiving CHO first. By chance, ten participants received KE+CHO first and four participants received CHO first demonstrating a distribution that did not unfairly bias our results, given the expected learning effect (performance on the second visit was 6% ± 9% better than that of the first, $p = 0.512$) and would only serve to underestimate the effect size of the KE+CHO treatment.

3.3.4 80 rpm endurance test

To assess the impact of ketone supplementation on patients' exercise performance, participants came into the lab in an "off" medication and overnight fasted (> 8 hours) state at either 8:00 or 10:00 am, similar to the baseline testing procedure. At 30 minutes prior to exercise testing, participants had βHB, glucose, and lactate levels measured by fingerstick (FreeStyle Precision Neo and Lactate Scout + blood analyzers). A new lancet was used to draw blood from either the index or middle finger and first drop blood was discarded prior to measurements. Immediately thereafter, participants received either the KE+CHO drink or the taste-matched CHO drink.

Thirty minutes after receiving the study drink (KE+CHO or CHO), participants again had βHB, glucose, and lactate levels measured before beginning the test. This time point was chosen based on previous data showing that the KE given at a similar dose to healthy participants reaches peak blood levels at between 30 and 60 minutes [58]. The beginning of this time window was selected as a measuring point such that participants would have either stable or still increasing βHB levels when they began exercise testing and maximum βHB would be available for oxidation. The endurance test was conducted on the same electronically braked cycle ergometer and began with the same 4-minutes warmup as in the baseline test, before the power was raised to each participant's personalized fixed wattage. Participants were verbally encouraged to maintain 80 rpm until failure, defined as the point at which the participant could not sustain a cadence of >70 Watts for a cumulative total of 20 seconds or s/he chose to stop (Figure 3.1).

In addition to the primary variable of interest, endurance performance (the duration of time participants could sustain 80 rpm), we also measured ventilation rate (V_E), oxygen consumption rate (VO_2), VO_{2Peak}, carbon dioxide exhalation rate (VCO_2), and respiratory exchange ratio (RER), calibrated to room O_2 and CO_2 concentrations, using the Metalyzer 3B cardiopulmonary monitoring system. We carefully distinguish VO_{2Peak} (the highest VO_2 obtained during the endurance test) from the standard nomenclature, VO_{2Max}, because the latter definitionally necessitates an increasing exercise load, which was not possible given the target primary outcome variable and parameters of this study design. Nevertheless, VO_{2Peak} and VO_{2Max} should strongly correlate, particularly because participants reached $VO2_{Peak}$ shortly before exercise failure. Exergy Expenditure (EE) and Oxygen economy (OE), defined as oxygen intake per unit power output, were subsequently calculated using formulas from Moseley et al. [59].

3.3.4 One-minute anaerobic threshold test

After the 80 rpm endurance exercise test, participants rested for approximately 5 minutes until their heart rates stabilized and returned to near baseline and they self-reported that they felt fully recovered. Participants then engaged in a one-minute anaerobic threshold test. This was included for two reasons. Firstly, to determine whether ketone supplementation could affect the maximum sustainable cadence PD subjects could maintain over a short period, which may reflect alterations in afferent stimulation of motor units (i.e., an altered capacity for the central nervous system to stimulate muscular contraction). Secondly, one-minute maximum intensity exercise is highly reliant on lactic glycolysis [60]. Thus, we used this test to functionally assess whether KE supplementation might suppress glycolysis in PD subjects. Participants were instructed to cycle at as high a cadence as possible at their personalized wattage for 60 seconds. Cadence was recorded

every second and average cadence was evaluated as the dependent variable. Immediate post-exercise (within 60 seconds) βHB, glucose, and lactate levels were then measured. The exercise testing protocol, including both the endurance and anaerobic threshold tests, are shown in Figure 3.1.

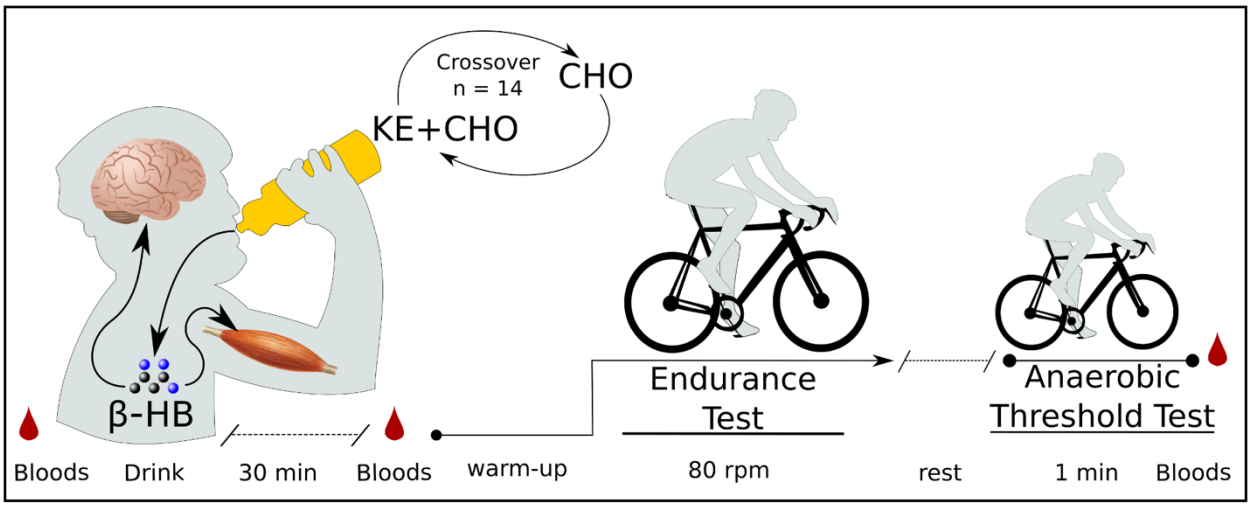


Figure 3.1 Exercise test protocol

N = 14 participants came into the lab on two separate testing days on which they received either a ketone ester plus carbohydrate (KE+CHO) or an isocaloric carbohydrate (CHO) drink in a randomized crossover fashion. Before drink consumption, before exercise (30 minutes after drink consumption), and following exercise, bloods were measured by fingerpick. Exercise testing occurred on a fixed-Watt cycle ergometer. Exercise began with a 4-minute warmup, after which wattage was increased to each participant's personalized fixed wattage set by baseline testing. For the endurance test, participants cycled at 80 rpm until failure. For the anaerobic threshold test, participants cycled at as high a cadence as possible for one minute.

3.3.5 Statistical analyses

Sample-size calculations were based on a report that a 12.6 rpm increase in cadence, from 66.0 to 78.6 rpm, translated into clinically meaningful differences in PD motor symptoms [54]. Although our study instead explored the duration of time at which participants could hold this presumed-to-be-therapeutic ~80 rpm cadence, these were the most relevant human data available upon which to base our calculation. To obtain 80% power with a significance level of 0.05, and allowing for 10% variance (6.6 rpm [54]) in participant performance, we calculated 12 participants would be required to detect a change in the primary outcome variable of endurance at 80 rpm. Two more participants were included to compensate for dropouts, bringing the total to 14 participants.

Blood data were analyzed using a two-way repeated measures ANOVA with Sidak corrections applied for multiple comparisons; respiratory measurements were analyzed using two-sided paired sample t-tests, and, because the performance data were not normally distributed, exercise performance was analyzed using Wilcoxon test. Differences were considered significant at $p < 0.05$. Values are reported as means ± standard error.

3.4 Results

3.4.1 Participant characteristics

The ten male and four female participants who completed the study all had early stage PD. Mean disease duration was 4.9 years and no participant had disease advanced beyond Hoehn and Yahr stage 2. All were between the ages 48 and 71 years, non-obese, and did not possess diagnoses or injuries, other than PD, that would influence their capacity to exercise (Table 3.1).

3.4.2 Blood β-hydroxybutyrate, glucose, and lactate

The KE+CHO drink increased participants' βHB levels from 0.1 ± 0.0 to 3.5 ± 0.3 mmol/L within 30 minutes of consumption (Table 3.2). Following exercise, βHB had decreased to 3.0 ± 0.3 mmol/L, reflecting the competing effects of βHB oxidation by skeletal muscle during cycling [35] and the continued liberation of βHB from the KE in the gut and liver [58]. No increase in βHB was observed in the CHO control group.

Glucose rose by ~1.7 mmol/L less in the KE+CHO group than in the CHO control (Table 3.2). While this could be explained simply be the fact that, as the calorie content of the drinks was the same, the control drink necessarily contained more carbohydrate, Mota AS et al. (manuscript under review) showed that KE decreases hepatic gluconeogenesis in patients with type 2 diabetics and that dysfunctional gluconeogenesis is largely responsible for poor glycemic control in type 2 diabetes, a common comorbidity of PD [61].

	Pre-Drink		Pre-Exercise (30 min After Drink)		Post-Exercise	
	CHO	KE+CHO	CHO	KE+CHO	CHO	KE+CHO
β-HB (mmol/L)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	3.5 ± 0.3 *	0.1 ± 0.0	3.0 ± 0.3 *
Glucose (mmol/L)	4.8 ± 0.1	4.7 ± 0.1	8.8 ± 0.6	7.0 ± 0.4 *	7.1 ± 0.8	4.3 ± 0.2 *
Lactate (mmol/L)	3.2 ± 0.5	4.0 ± 0.6	2.8 ± 0.3	2.6 ± 0.4	8.5 ± 0.7	7.4 ± 0.6

Table 3.2 Blood β-hydroxybutyrate, glucose, and lactate

Participants' blood β-hydroxybutyrate (βHB), glucose, and lactate levels ± standard error, as measured by fingerstick, prior to study drink ingestion, prior to exercise (30 minutes after study drink ingestion), and following exercise. P-values for between treatment differences were calculated using a two-way repeated measures ANOVA. * indicates $p<0.001$ for the comparison between the carbohydrate (CHO) and ketone ester plus carbohydrate (KE+CHO) drinks at each timepoint. Data include n = 13 participants. One participant on a self-prescribed ketogenic diet with β-hydroxybutyrate levels >2 mmol/L was excluded from data summarized in this table because her data were an outlier and skewed the β-hydroxybutyrate values. However, inclusion or exclusion of this participant's data from this or any data set presented in this study does not impact statistical significance.

3.4.3 Ketone ester improved endurance performance and did not affect maximum sustainable cadence

Participants cycled for 24% ± 9% ($p = 0.027$) longer at the therapeutic target of 80 rpm [53, 54] after consuming the KE+CHO drink compared to the isocaloric CHO control (Figure 3.2). Absolute time sustained on the KE+CHO and CHO arms were 13:46 ± 2:50 and 11:58 ± 2:43 minutes, respectively. The KE+CHO drink did not negatively impact maximum sustainable cadence, as measured on the one-minute anaerobic threshold test (absolute values: 113 ± 3 rpm on KE+CHO vs. 110 ± 4 rpm on CHO).

VO_2 and VO_{2Peak} did not increase significantly ($p = 0.08$ and 0.07, respectively), whereas total V_E increased by >4 L/min. There was no change in VCO_2, EE, and OE between the groups.

As expected, given that βHB has a respiratory quotient (RQ) of 0.89 [62] and increases intramuscular triglyceride (RQ = 0.70) oxidation [35], RER was significantly decreased in the KE+CHO arm relative to the CHO control arm, in which carbohydrate (RQ = 1.00) was the presumptive primary fuel. Mean rpm did not differ between the groups and so did not confound the results (Table 3.3).

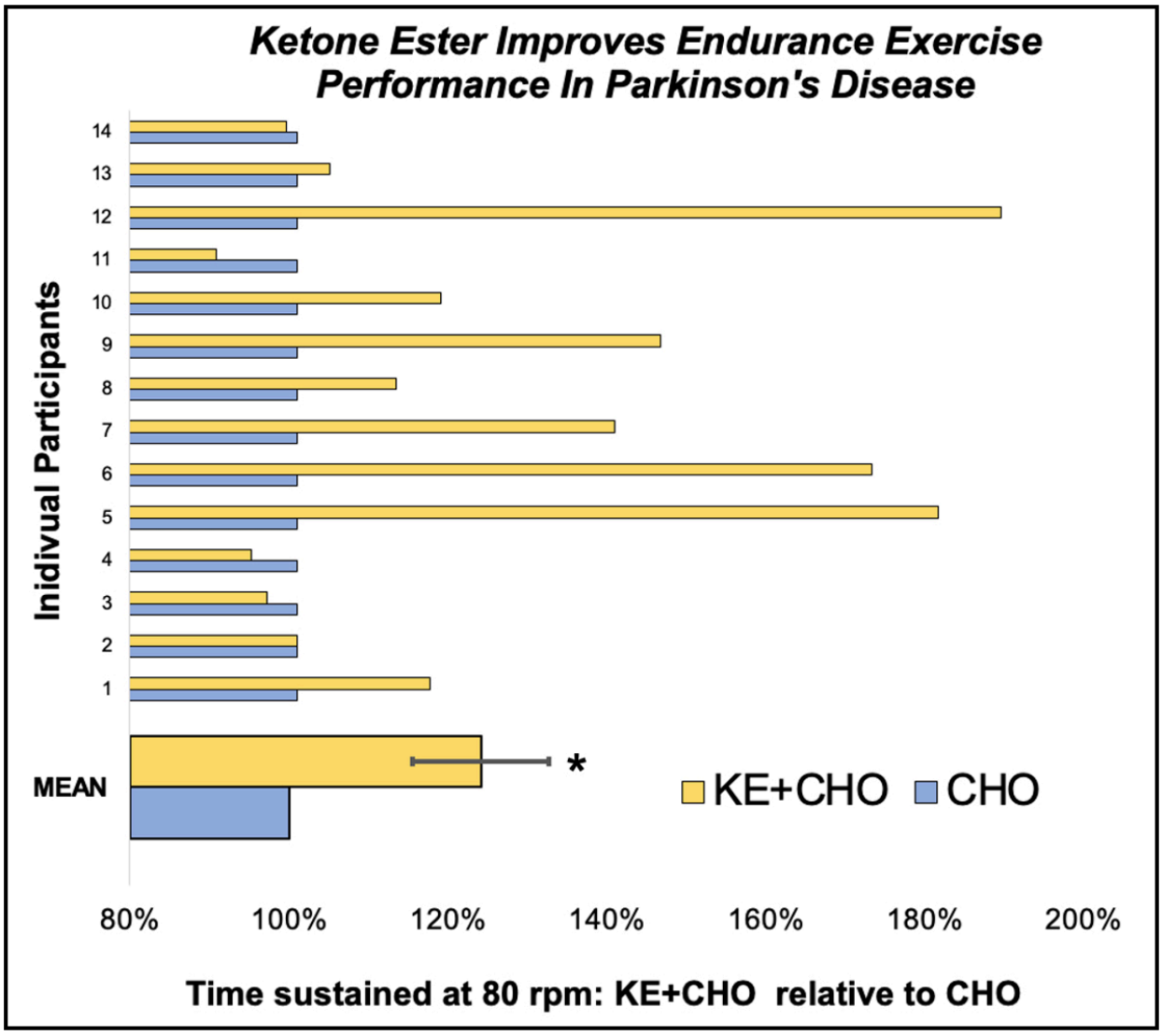


Figure 3.2 Endurance exercise test performance

On the endurance test, participant cycled for 24% ± 9% longer at 80 rpm following the ketone ester plus carbohydrate (KE+CHO) drink relative to performance following the isocaloric carbohydrate (CHO) drink. Data are expressed as performance following the KE+CHO drink divided by performance following the CHO drink, where performance is defined as the length of time participants could sustain 80 rpm. * indicates $p = 0.027$ as determined by Wilcoxon test.

	RER	V_E (L/min)	VO_2 (L/min)	VO_{2Peak} (L/min)
CHO	0.954 ± 0.012	47.42 ± 3.53	1.386 ± 0.121	1.81 ± 0.14
KE+CHO	0.931 ± 0.011	51.67 ± 4.52	1.502 ± 0.133	1.89 ± 0.15
P-value	0.03 ↓	0.03 ↑	0.08 ↑	0.07 ↑
	VCO_2 (L/min)	EE (kCal/hr)	OE (mL/Watt)	Cadence (rpm)
CHO	1.410 ± 0.113	422.9 ± 35.5	0.30 ± 0.05	78.06 ± 0.78
KE+CHO	1.415 ± 0.129	450.0 ± 40.1	0.31 ± 0.04	77.76 ± 0.82
P-value	0.92	0.12	0.35	0.40

Table 3.3 Respirometry data

Participants' respiratory exchange ratio (RER), ventilation rate (V_E), oxygen consumption rate (VO_2), VO_{2Peak}, carbon dioxide expiration rate (VCO_2), energy expenditure (EE), oxygen economy (OE), and mean revolutions per minute (rpm) ± standard error on the endurance exercise test. P-values were calculated using two-tailed paired sample t-tests and arrows indicate the significant or trending directional effects of the ketone ester plus carbohydrate (KE+CHO) drink relative to the isocaloric carbohydrate (CHO) control.

3.5 Discussion

3.5.1 Ketone-induced improvements in endurance

Consumption of a KE+CHO nutritional supplement increased, by 24% ± 9%, the time individuals with PD sustained an 80 rpm cycling cadence, relative to their performance following an isocaloric CHO control drink. At a systems level, the associated changes, or lack thereof, in our secondary outcome variables (in particular, βHB, RER, VO_2, VO_{2Peak}, VCO_2, and lactate) were consistent with those thought to enhance endurance performance in athletes.

As expected, a single KE drink significantly increased βHB to >3mmol/L, ketosis equivalent to that observed after several days of fasting and one difficult to achieve even on the strictest ketogenic diet. The ketotic state achieved by the study participants explains the observed decrease in RER. Compared to CHO, which has an RQ of 1.00, βHB has an RQ of 0.89 [62]. Furthermore, ketosis induced by KE+CHO drink ingestion increases the oxidation of intramuscular triglycerides (RQ of 0.70) during endurance exercise [35]. Thus, βHB oxidation and a shift to fat catabolism can explain the decrease in RER.

As RER is defined as the ratio VCO_2/VO_2 (and more O_2 is oxidized during fat oxidation compared to glucose oxidization because fatty acids are a more reduced substrate [62]), it makes sense that we observed a trend toward increasing VO_2 without any trend in VCO_2. Concordant with VO_2, VO_{2Peak} too trended upwards in the KE+CHO group relative to the control, although did not achieve statistical significance, likely because of the limited sample size. (Assuming intraindividual variance equal to the effect size we observed for these trending secondary variables, 16 participants, rather than 14, would be required to obtain 80% power at a significance level of

0.05.) Nevertheless, the putative 80 mL/min increase in VO_{2Peak} is notable because VO_{2Max} (see methods for distinction) is a marker of cardiovascular fitness, one of the best predictors of longevity [63], and has been associated with elevated cognitive and motor scores in PD [64]. Admittedly, VO_{2Peak}, as measured in this acute intervention study, did not reflect an improvement in participants' overall fitness; however, by increasing the ability of persons with PD to sustain endurance exercise, a KE supplement could improve fitness and true VO_{2Max}. An interesting qualification to this prediction is that persons with PD exhibit impaired metabolic adaptations in VO_{2Max} and V_E in response to routine aerobic exercise [65]. Therefore, it's possible that increasing endurance may not translate into improved fitness even with training. Alternatively, it's also possible that KE could help to compensate for systemic metabolic dysfunction in persons with PD to rescue their ability to adapt to aerobic exercise (see discussion of "β-oxidation insufficiency" and CI blockade" below).

The lack of change in VCO_2, despite an increase in overall V_E, is itself notable when one considers the metabolism of the KE drink. The butanediol component of the KE is metabolized in the liver to form a keto-acid, which lowers blood pH from 7.41 to 7.31 within one hour of KE ingestion [58]. As blood acidification drives a rightward shift in the oxygen-hemoglobin dissociation curve via the Bohr effect, the moderate acidification could improve the efficiency of O_2 unloading at skeletal muscles and contribute to an increase in VO_2 and VO_{2Peak} [66] (Figure 3.3i). Blood acidification and O_2 unloading also increase the affinity of hemoglobin for CO_2 by the Haldane effect (the converse of the Bohr effect), which increases CO_2 binding to hemoglobin and carbaminohemoglobin formation [67]. Furthermore, blood acidification inhibits carbonic

anhydrase activity [68]. This predicts that KE ingestion could cause mild CO_2 sequestration during exercise, perhaps driving up V_E.

The trend to lower lactate levels post-exercise following KE+CHO ingestion, as compared to CHO ingestion, is also worth remarking upon in the context of the literature. Lactate levels were higher at baseline in our participants than one would expect of individuals without PD, which is consistent with research demonstrating ~50% elevated resting lactate in patients with PD and metabolically similar conditions (multiple sclerosis and mitochondrial complex I (CI) deficiency) relative to healthy controls [56, 69-71]. The high baseline lactate may be explained by the 16-54% decreased peripheral CI activity in PD patients [25, 72-77], including in skeletal muscle [78, 79]. We refer to this as the "CI blockade." Impaired mitochondrial respiration would force a relative shift towards glycolysis, generating more lactate. KE+CHO supplementation in elite athletes diminishes lactate accumulation during exercise by ~50% by shifting metabolism from glycolysis towards oxidative phosphorylation [35] (Figure 3.3ii). Consistent with these prior findings, in the present study we observed the same trend towards a blunted lactate spike following the KE+CHO drink.

The changes in βHB and RER (as well as the noted trends in VO_2, VO_{2Peak}, and lactate, for which this study was not sufficiently powered nor was it intended to be) are consistent with the literature, and predict that the KE+CHO drink would improve endurance exercise performance in PD, as we observed. However, the above-mentioned mechanisms may not fully explain the 24% effect size.

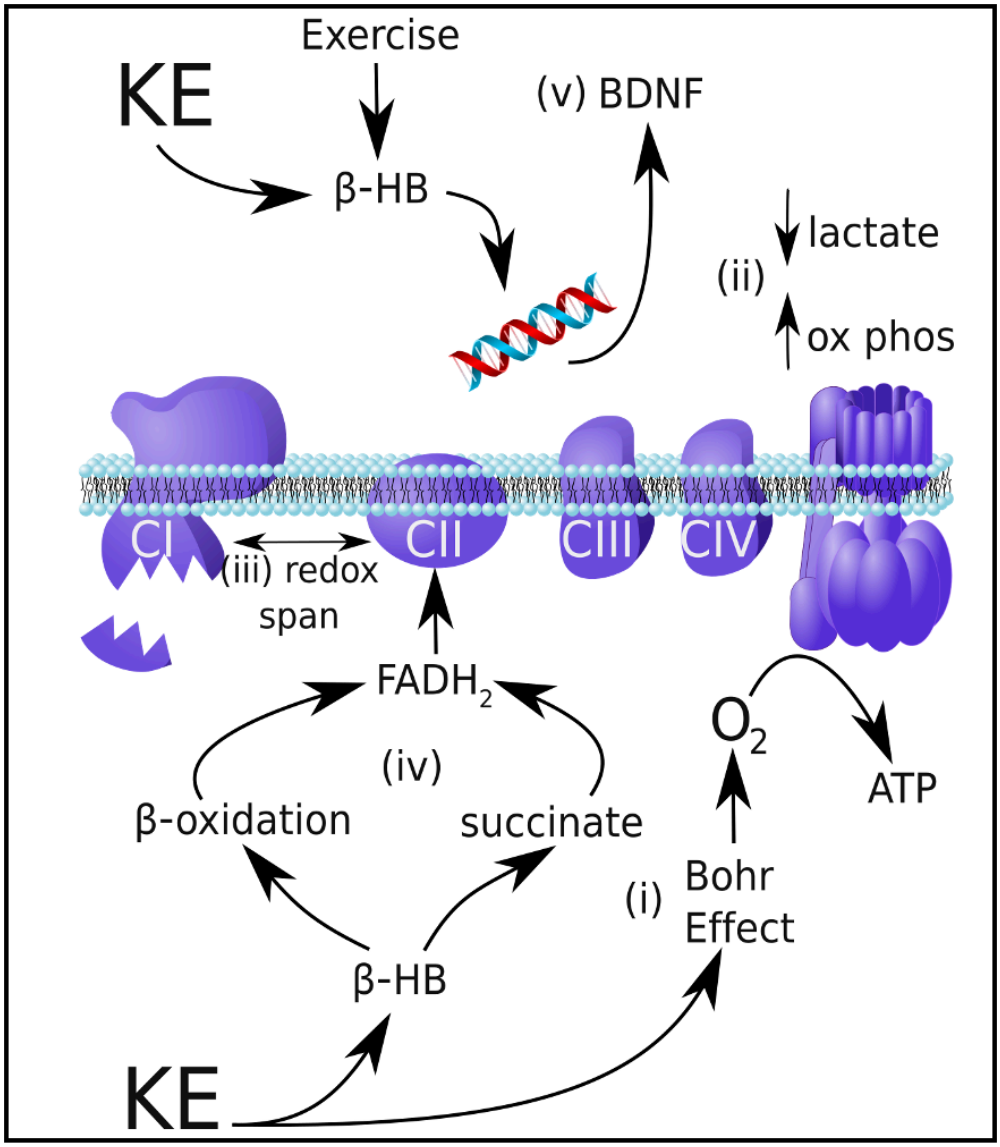


Figure 3.3 Possible mechanisms by which a ketone ester can improve endurance performance and provide neuroprotection in Parkinson's disease

(i) By safely shifting blood pH to induce the Bohr Effect, the ketone ester (KE) supplement can increase oxygen delivery to skeletal muscle and increase VO_2. (ii) βHB shifts exercise metabolism towards mitochondrial oxidative phosphorylation during exercise relative to glycolysis, reducing lactate production. (iii) In mitochondria, βHB can increase the redox span of the respiratory chain, thereby increasing the efficiency of energy production by oxidative metabolism. (iv) In Parkinson's disease, complex I (CI) is damaged and underactive, whereas complex II (CII) is entirely functional. By increasing β-oxidation of intramuscular triglycerides and increasing succinate, βHB can increase CII flux and circumvent the CI blockade. (v) In addition to reducing oxidative stress and inflammation (not shown), βHB produced during exercise inhibits HDACs and activates CREB/CRP signaling to upregulate neuroprotective BDNF expression.

3.5.2 Mitochondrial efficiency and enhanced endurance

A general cellular explanation for how the KE+CHO drink improved endurance performance in our participants is that the βHB component of the drink improved mitochondrial efficiency. βHB has the potential to increase ATP production, and/or the free energy liberated by ATP hydrolysis, by exerting opposite redox effects on the respiratory chain electron carriers, NADH and CoQ10 [80]. This mechanism of increasing redox potential energy within the mitochondrial respiratory chain is analogous to increasing gravitational potential energy by lifting a bowling ball "electron" higher from the ground [30]. Stated concisely, ketosis may increase the redox span of the electron transport chain to increase the efficiency of mitochondrial metabolism (Figure 3.3iii). This mechanism is relevant to, but not specific to, PD.

PD is characterized by an increased dependence on carbohydrate, relative to fat, as fuel. Early work by Landin et al. showed that individuals with PD deplete glycogen during exercise at four-fold the rate of control subjects and exhibit fatty acid turnover [56]. This impaired fatty acid metabolism has been confirmed more recently by Saiki et al., who showed that β-oxidation is impaired early in PD, independent of levodopa dose. They termed this phenomenon Parkinson's "β-oxidation insufficiency" [15]. We have shown previously that KE shifts metabolism to increase the β-oxidation of intramuscular triglycerides in athletes [35]. If this phenomenon is generalizable to PD, a correction of "β-oxidation insufficiency" could contribute to the large effect size of KE on endurance performance reported in this study.

Furthermore, as mentioned above, PD is metabolically characterized by the mitochondrial "CI blockade." CI dysfunction is not simply localized to the brain, but extends to the periphery, including to skeletal muscle [25, 72-79]. As electrons can enter the respiratory chain at either CI or complex II (CII), and CII function does not appear to be impaired in PD [79, 81], interventions that promote CII-flux over CI-flux would be predicted to enhance mitochondrial metabolism in PD.

By increasing β-oxidation and also by increasing succinate flux, as compared to glucose, the KE drink provided to participants in this study should preferentially increase CII activity to improve mitochondrial metabolism in PD (Figure 3.3iv). With regard to β-oxidation, each cycle of β-oxidation generates 1 $FADH_2$: 1 NADH, as compared to each Krebs cycle, which generates 1 $FADH_2$: 3 NADH. Thus, the oxidation of fatty acids by β-oxidation and the Krebs cycle generates a higher $FADH_2$: NADH ratio than the oxidation of glucose and shifts electron flux towards CII. (As an example, the oxidation of C18:0 stearic acid, through eight cycles of β-oxidation and nine Krebs cycles, generates a $FADH_2$: NADH ratio of 16:32 or 0.5, whereas oxidation of glucose yields an $FADH_2$: NADH ratio of 2:10 or 0.2.) With regard to succinate flux, the rate limiting step of βHB catabolism generates succinate, which is used to reduce FAD to $FADH_2$; and, thus, succinate serves as an oxidative fuel for CII. Beyond mechanistic speculation, Tieu et al. showed that administration of βHB to MPTP-treated PD mice protected dopaminergic SNpc neurons from cell death and that this effect is blocked by the specific inhibition of CII [81].

There is, therefore, a mechanistic and animal model basis for hypothesizing that KE improves endurance performance in PD, specifically, by helping to correct "β-oxidation insufficiency" and circumvent the "CI blockade."

3.5.3 Ketone ester holds promise as an indirect disease-modifying therapy for Parkinson's disease

By improving patients' abilities to engage in therapeutic 80 rpm cycling, KE could indirectly provide disease-modifying neuroprotection in PD. Earlier, we mentioned exercise paradox for PD: vigorous exercise may slow disease progression, but as the disease progresses patients lose the ability to engage in vigorous exercise. If our data are replicated in larger and longer-term studies, patients of the future could supplement with KE prior to exercise in order to engage in more vigorous more effective exercise over longer periods of time. KE supplementation could eliminate, or at least help compensate for, the exercise paradox, thereby providing patients with a tool to improve their disease trajectory.

3.5.4 Ketone ester holds promise as a direct disease-modifying therapy for Parkinson's disease and may synergize with exercise

This study was an acute intervention that focused on using KE as an adjunct to boost the efficacy of exercise therapy. However, it is important to note that the βHB molecule, upon which KE is based, has direct disease-modifying potential in PD. As we have previously described how βHB can address the foundational metabolic dysfunctions that underly to PD: energetic dysfunctions, oxidative stress, inflammation, and cell death [30], we here specifically elaborate on how βHB and exercise may work together to protect against and slow the progression of PD, through mechanisms involving neurotrophic factors.

Work on animal models of PD has demonstrated that part of the neuroprotection afforded by endurance exercise is attributable to BDNF and GDNF, two neurotrophic factors that are reduced in the SNpc of human PD patients by as much as 20% per neuron, relative to healthy age-matched controls [48]. In independent studies, aerobic exercise protected rats from motor deficits induced by direct injection of 6-OHDA into the striatum. The preservation of motor function in the exercised rats was associated with roughly 60% increases in BDNF and GDNF, and further with the preservation of dopaminergic SNpc neurons [42, 46]. Other studies using MPTP rodent models of PD support these findings [43, 82, 83] and provide encouragement that exercise is neuroprotective in PD.

The BDNF/GNDF neuroprotective mechanism directly implicates βHB. *In vitro,* βHB induces neuronal BDNF expression [84, 85], and inhibition of the BDNF receptor diminishes the neuroprotective benefits of βHB [84]. *In vivo*, in mice, 6 weeks of exercise increased βHB and BDNF levels in the brain [85]. Other data validate and elaborate upon this "exercise-βHB-BDNF/GNDF" model. Sleiman et al. found that exercise increased βHB in mice and that βHB induces BDNF in primary cortical neurons by acting as a histone deacetylase (HDAC) inhibitor and decreasing HDAC binding to the BDNF promotor [47]. What's more, exercise and direct intraventricular delivery of βHB were each sufficient to increase BDNF expression in the brain [47].

βHB appears to act more generally as an epigenetic modifier to increase BDNF expression. Not only does βHB block histone deacetylation to increase BDNF expression, but Hu et al. showed that βHB actively upregulates BDNF promoter acetylation by inducing cAMP response element-binding protein (CREB)/CREB-binding protein (CBP) signaling [86]. Therefore, by the complementary mechanisms of downregulation of deacetylation and upregulation of acetylation (along with other putative mechanisms such as inducing histone demethylation [86] and adaptive hormetic oxidative stress [84]), βHB can increase BDNF expression to support neuron survival (Figure 3.3v).

Critically, in the *in vivo* studies conducted by Hu et al., the dose of βHB administer to mice (120 mg/kg/day) that increased BDNF expression was 18-times less than the dose of ΔG® KE drink that has been validated as safe in humans (2.142g/kg/day) [87]. Therefore, there is at least early animal evidence in support of the notion that exogenous ketone supplements, and specifically the ΔG® KE drink used in this study, would exert neuroprotective benefits in PD, not only indirectly, by increasing exercise performance, but also directly, by increasing BDNF. This is an area ripe for future investigations.

3.5.5 Limitations

Our study has several limitations. First, PD symptomology is highly variable day to day. Therefore, although our crossover design allowed participants to serve as their own controls, intrapersonal variability was an unavoidable complicating factor. Second, we chose to test participants in an "off" medication state so as to isolate the impact of the intervention. As the KE supplement holds potential primarily as an adjunct to, and not a replacement for, dopaminergic therapy, future studies should examine the impact of the KE drink when patients are "on" medications. Third, while our study was sufficiently powered to achieve statistical significance with respect to the main outcome variable, a larger sample size may have allowed us to report significant differences for VO_2, VO_{2Peak}, and lactate, which are consistent with previous literature and are relevant to the mechanistic understanding of why the KE+CHO drink enhanced performance. Fourth, this study was aimed only at investigating the acute effects of ketosis on exercise performance in PD, and therefore excludes the potentially larger neuroprotective benefits derived from chronic ketosis.

3.5.6 Future directions

Larger scale studies are required to validate these results. Future studies should examine the mechanisms of any effect, specifically the possibility that the KE corrects Parkinson's "β-oxidation insufficiency" and compensates for the "CI blockade." Finally, it would be invaluable to conduct rigorous large-scale prospective studies on the hypothesized protective benefits of ketogenic interventions in the coming decades in order to properly assess whether exercise and ketosis, independently and together, can be used as true disease modifying treatments for PD.

Chapter 4. *Effects of a ketone ester drink on brain metabolism in Parkinson's disease as measured by* ^{31}*P-MRS*

4.1 Abstract

Parkinson's disease is associated with decreased ATP levels, particularly in affected regions such as the substantia nigra. β-hydroxybutyrate is an alternative fuel substrate for neurons that exhibit impaired glucose metabolism. Previous *in vitro* work in Parkinson's disease models suggests β-hydroxybutyrate can increase ATP production and ameliorate further neuron cell death. This study was designed to investigate whether a single dose of ketone ester could increase ATP levels one hour post drink in a population of eleven patients with Parkinson's disease, as measured by 31 phosphorus-magnetic resonance spectroscopy. No significant change in ATP levels, nor in the levels of other measured metabolites was detected. The negative results reported in this acute administration study do not preclude the possibility that chronic ketosis could be neuroprotective in Parkinson's disease and other neurodegenerative disorders, an important distinction and concept that is elaborated upon in the discussion.

4.2 Background

4.2.1 Parkinson's disease is caused by the loss of energetically demanding dopaminergic neurons

Parkinson's disease (PD) is biologically characterized by the death of dopaminergic neurons within the midbrain's substantia nigra (SN). The death of these dopaminergic neurons is thought to be caused, at least in part, by errors in mitochondrial metabolism that prevent SN neurons from generating sufficient energy to sustain function. Physiologically, it makes sense that dopaminergic neurons would be among the most susceptible to energy deficit, as they are among the most energetically demanding neurons in the brain owing to their large size, extensive degree of arborization, and unique calcium pacemaking activity [8, 88]. Prior work has confirmed that the presumed energy crisis in the PD brain is associated with a 44% decrease in ATP levels in the midbrain compared to age-matched controls, as measured by 31 phosphorus-magnetic resonance spectroscopy (^{31}P-MRS) [36].

4.2.2. β-hydroxybutyrate could rescue energy metabolism in neurons to slow neuronal loss

β-hydroxybutyrate (βHB) has the potential to rescue dopaminergic neurons from energy crisis to prevent or slow further dopaminergic neuron death and disease progression. There are at least two molecular rationales for this hypothesis. First the catabolism of βHB increases the redox span between the NAD^+/NADH and Q/QH_2 electron carrier couples in the respiratory chain (at least in βHB perfused rat hearts [80]), increasing the energy liberated by the transfer of electrons from NADH to Q and increasing the number of protons pumped into the intermembrane space that can be used to drive oxidative phosphorylation. Second, the rate limiting step in βHB catabolism generates succinate, a Krebs cycle intermediate and metabolic substrate that feeds electrons into the respiratory chain at complex II and, thereby, can circumvent the complex I blockade that occurs

in PD mitochondria [30, 81] (Figure 4.1). In *in vitro* and animal models of PD, βHB can increase ATP levels [81], increased ATP can protect PD neurons from energy-crisis induced death [89], and βHB can protect PD neurons from cell death [81, 90]. Therefore, this study investigated whether the βHB-based ΔG® ketone ester (KE) could increase ATP levels in the brains of persons with PD [36].

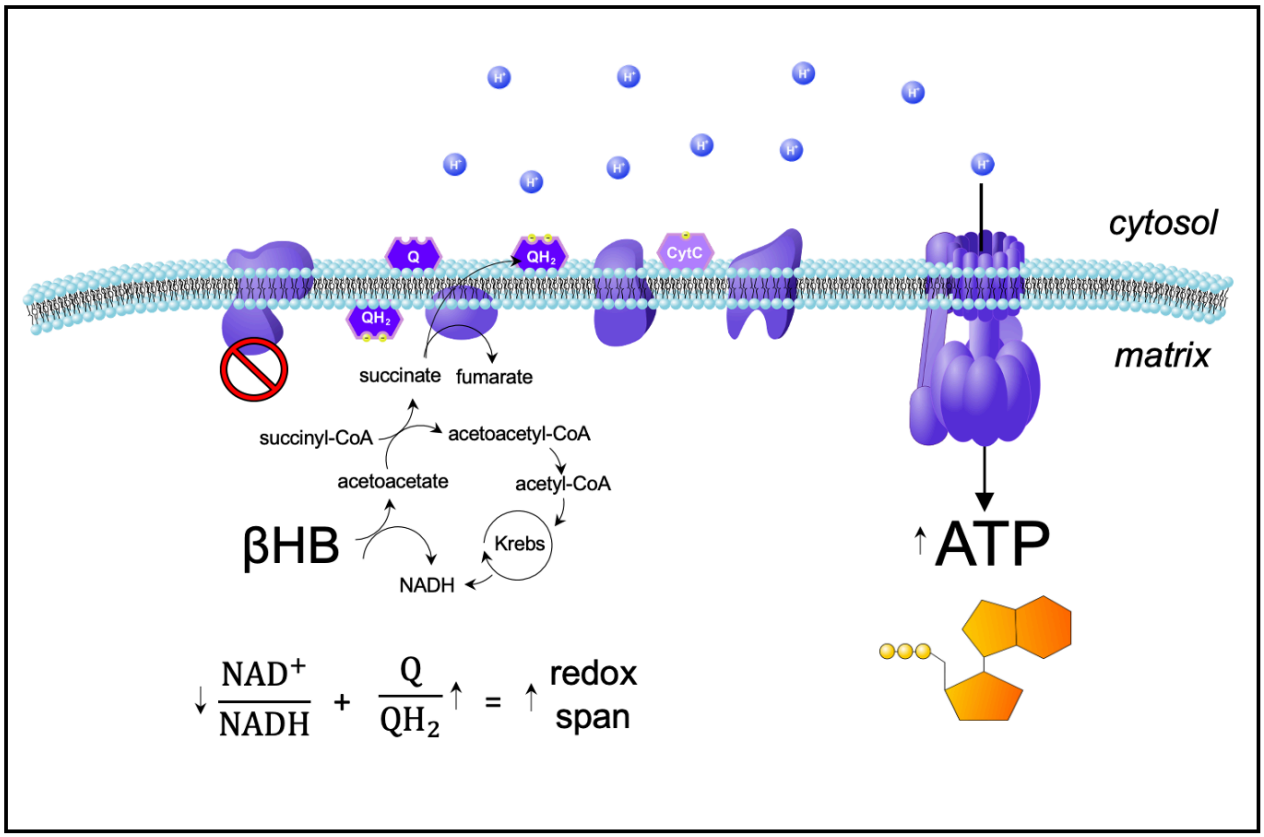


Figure 4.1 β-hydroxybutyrate could increase ATP production in PD neurons

βHB can increase the redox span between the NAD^+/NADH and Q/QH_2 couples and circumvent that complex I blockade. Thus, βHB, could increase ATP production in PD neurons and rescue cells from energy crisis.

4.2.3. β-hydroxybutyrate could alter NAD(H) metabolism in Parkinson's disease

In addition to energy crisis and ATP depletion, NAD(H) metabolism is of interest in PD. NAD^+ deficiency is associated with most neurodegenerative disorders, as well as with aging in general [91-94], and NAD^+ boosting therapies have been and are being explored for PD [95-98]. The notion that βHB could improve NAD^+ levels to exert long-term neuroprotection in PD, and other age-related neurodegenerative diseases, is backed by molecular logic. Glucose requires the glycolytic reduction of four (two cytosolic and two mitochondrial) NAD^+ to NADH to yield acetyl-CoA, whereas βHB only requires the reduction of one mitochondrial NAD^+ per equivalent to generate acetyl-CoA. Thus, ketosis and βHB should spare NAD^+, and particularly cytosolic NAD^+. Finally, 7T brain imaging suggests even light ketosis might may positively impact NAD(H) metabolism in the human brain [99]. Therefore, while it is not the primary objective of this study, it is relevant to consider NAD^+ metabolism in a discussion of ketosis and neurodegenerative disease.

4.3 Methods

4.3.1. Study overview

11 participants with PD who fufilled criteria set out in section 2.3 came into the Oxford Centre for Clincal Magneitc Responance Research (OCMR) at either 8:00 or 9:00 am before having had any medications, breakfast, or fluids other than water. Baseline βHB and glucose were measured by fingerstick, afterwhich participants underwent ^{31}P-MRS scans. After the baseline scans, 25 mL of ΔG® KE drink was administered to participants and they then waited for for one hour prior to their second scans. During this hour, participants were asked to rest in a waiting room and remain sedentary. They were permitted to read or use their smartphones. At the one hour mark, βHB and glucose levels were again drawn and participants underwent post-KE ^{31}P-MRS scans.

After all scans were completed fourteen brain regions were idenfified by consensus based on anatomical landmarks. Metabolites represented in the spectra for pre- and post-KE scans for each region for every participant were quantified as areas under the curve and adjustments to calculate absolute concentration of the measured metabolites were made by comparison to a phantom of known 31 phosphporus concentrations. The project workflow is shown in Figure 4.2 and further details on the scanning protocol are giving in the next section.

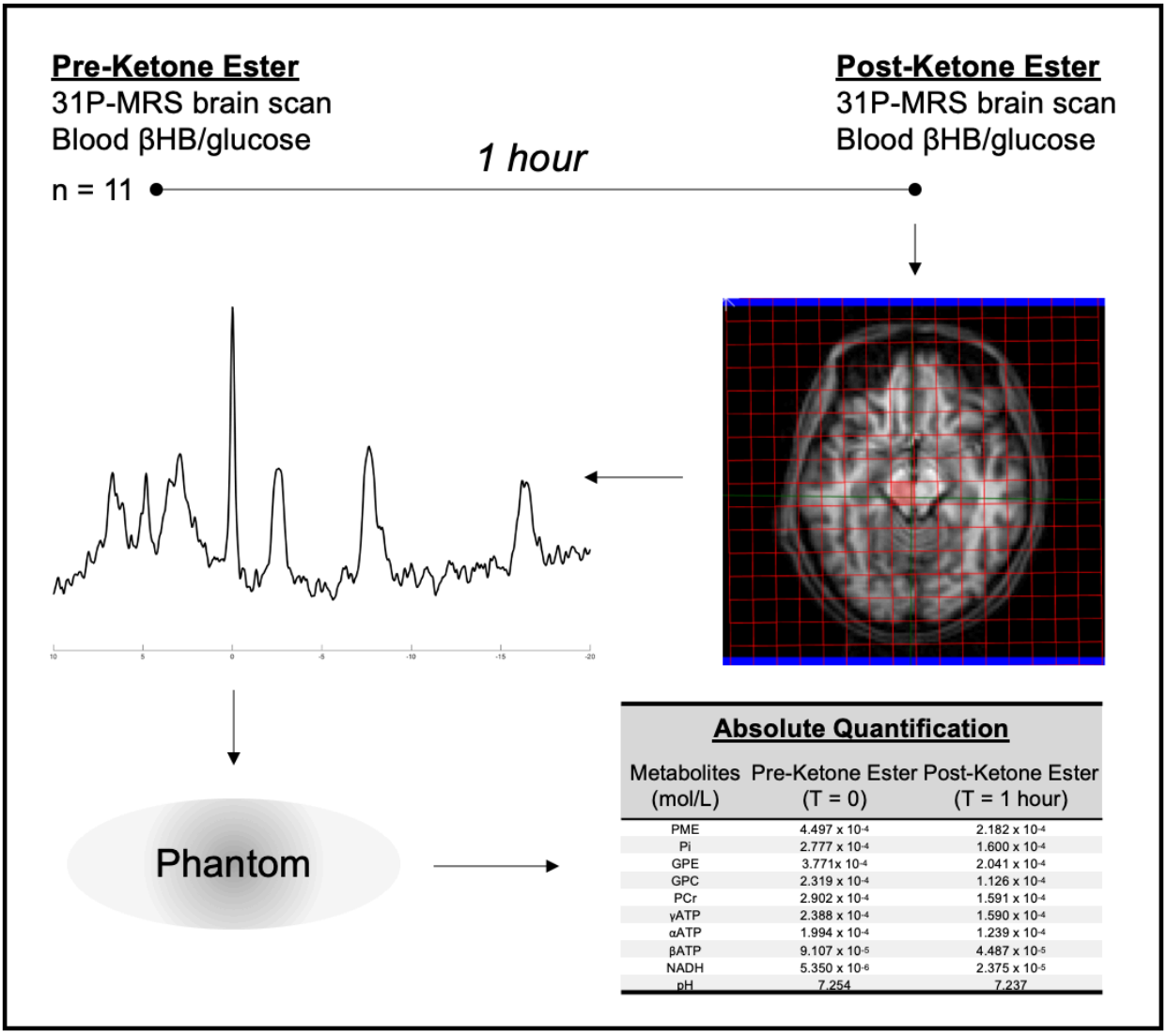


Figure 4.2 Protocol overview

11 participants underwent blood tests for βHB and glucose and a baseline ^{31}P-MRS scan immediately before consuming 25 mL of ketone ester. One hour later, we repeated participants blood and brain scan measurements. We identified each brain region of interest and quantified metabolites of interest in the pre- and post-ketone ester spectra. Finally, we scanned a phantom in order to obtain absolute quantifications for each metabolite in each scan for all brain regions.

4.3.2. ^{31}P-MRS scanning protocol

Brain scans on each participant were conducted using a 3T magnetic resonance scanner (Magnetom Trio, Siemens Healthcare, Erlangen, Germany) with a dual-tuned ^{1}H/^{31}P birdcage head coil (Rapid Biomedical, Rimpar, Germany). Based on a T1-weighted MRI localizer, a ^{31}P 3D ultrashort TE chemical shift imaging matrix (10 x 10 x 8 mm^3) was positioned to cover whole brain. Acquisition parameters were as follows: voxel size = 23 x 23 x 25 mm^3, field of view = 230 x 230 x 200 mm^3, TR = 1 second, flip angle = 40 degrees, bandwidth = 4 kHz, number of acquisition weighted averages = 32, total scan time = 23:40 minutes. In addition, a nonlocalized acquisition of a phenylphosphonic acid fiducial positioned at the coil was performed for data normalization purposes. For absolute quantification using the phantom replacement technique, the whole protocol was repeated on a 3 L cylindrical bottle filled with a known concentration of phenylphosphonic acid (14 mol/L).

Measured metabolites included phosphate monoester (PME), inorganic phosphate (Pi), glycerophosphoethanolamine (GPE), glycerophosphocholine (GPC), phosphocreatine (PCr), αATP, βATP, γATP, and NAD(H). Among ATP signals, βATP was of primary interest as the β phosphate in ATP is the only one flanked by two other phosphate groups. Thus, it possesses a unique chemical shift, as compared the α and γ phosphates in ATP that have chemical environments similar to those of α phosphate in ADP and β phosphate in ADP, respectively. Voxels corresponding to right and left dorsolateral prefrontal cortices, right and left temporoparietal cortices, right and left SN, right and left thalamus, right and left posterior lentiform nuclei, right and left caudate, occipital cortex, and cingulate cortex were selected by consensus. Representative photos of the right SN of participant 5 are shown in Figure 4.3.

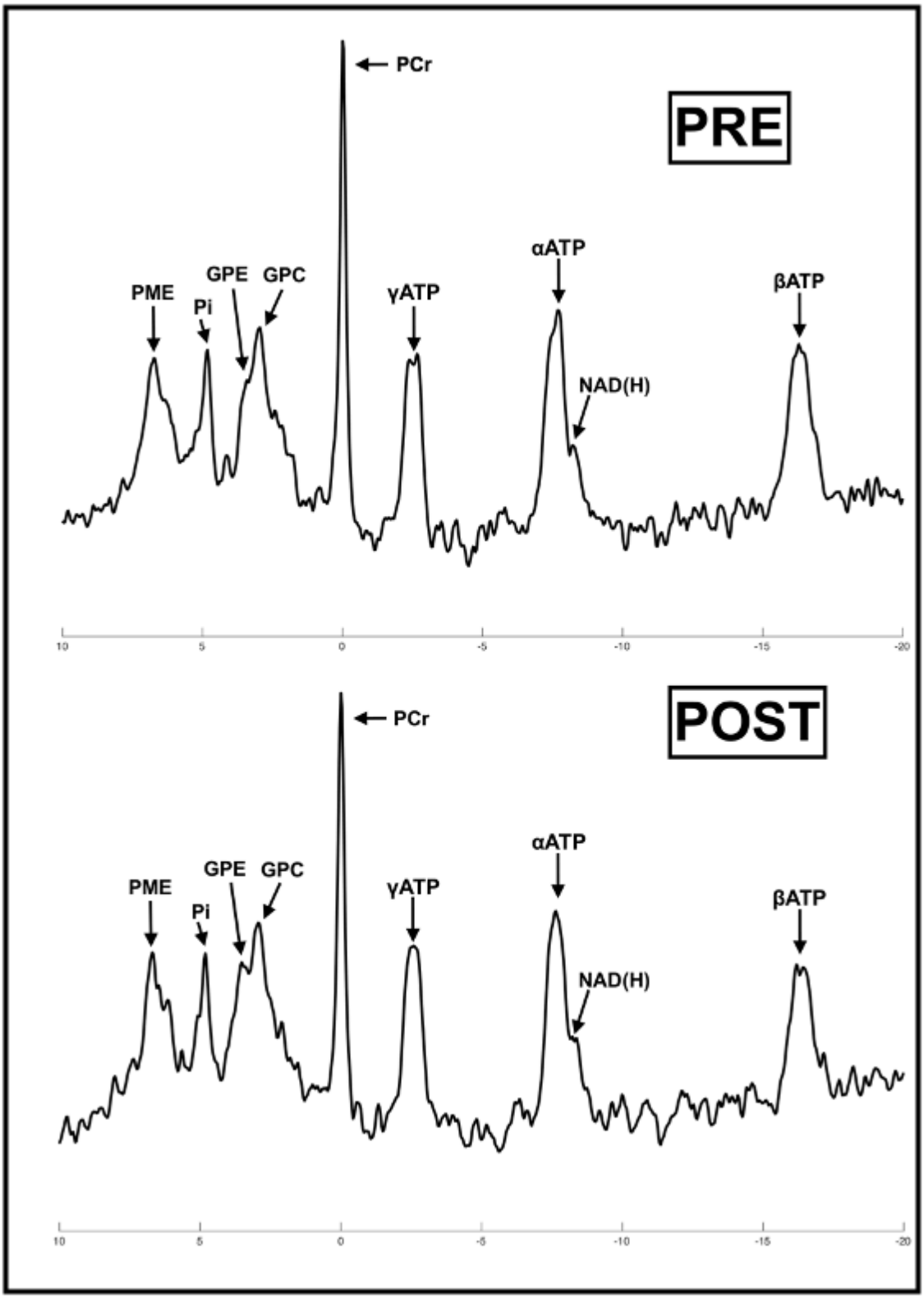


Figure 4.3 Representative spectra

Pre- and post-ketone ester spectra for the right substantia nigra of participant 5.

4.3.3. Pre- and Post-ketone ester statistical comparisons

Two-tailed paired sample t-tests with Bonferroni-correction for multiple comparisons were used to compare pre- and post-KE measures for the aforementioned metabolites. Bonferroni-correction for multiple comparisons compensates for the fact that, given an alpha threshold of 0.05, there was a one in twenty chance of type I error for each comparison among fourteen brain regions. Therefore, to retain the desired overall alpha of 0.05, this value was divided by the number of individual tests being performed to set the alpha threshold for each individual test. Thus, to retain an overall alpha threshold of 0.05, the alpha threshold for any individual brain region was 0.004.

Bonferroni-correction:

$$\frac{\text{overall } \alpha}{\text{number of tests}} = \text{individual } \alpha \quad : \quad \frac{0.05}{14} = p < 0.004$$

4.4 Results

4.4.1 Ketone ester increased β-hydroxybutyrate and decreased glucose in serum

All participants arrived at the lab in a fasted state with baseline βHB levels of ≤ 0.2 mmol/L. One hour following KE consumption, serum βHB levels increased by an average of 4.2 ± 0.3 mmol/L, ($p < 0.001$). Correspondingly, KE decreased participants serum glucose levels by 0.8 ± 0.2 mmol/L ($p < 0.001$) (Figure 4.4).

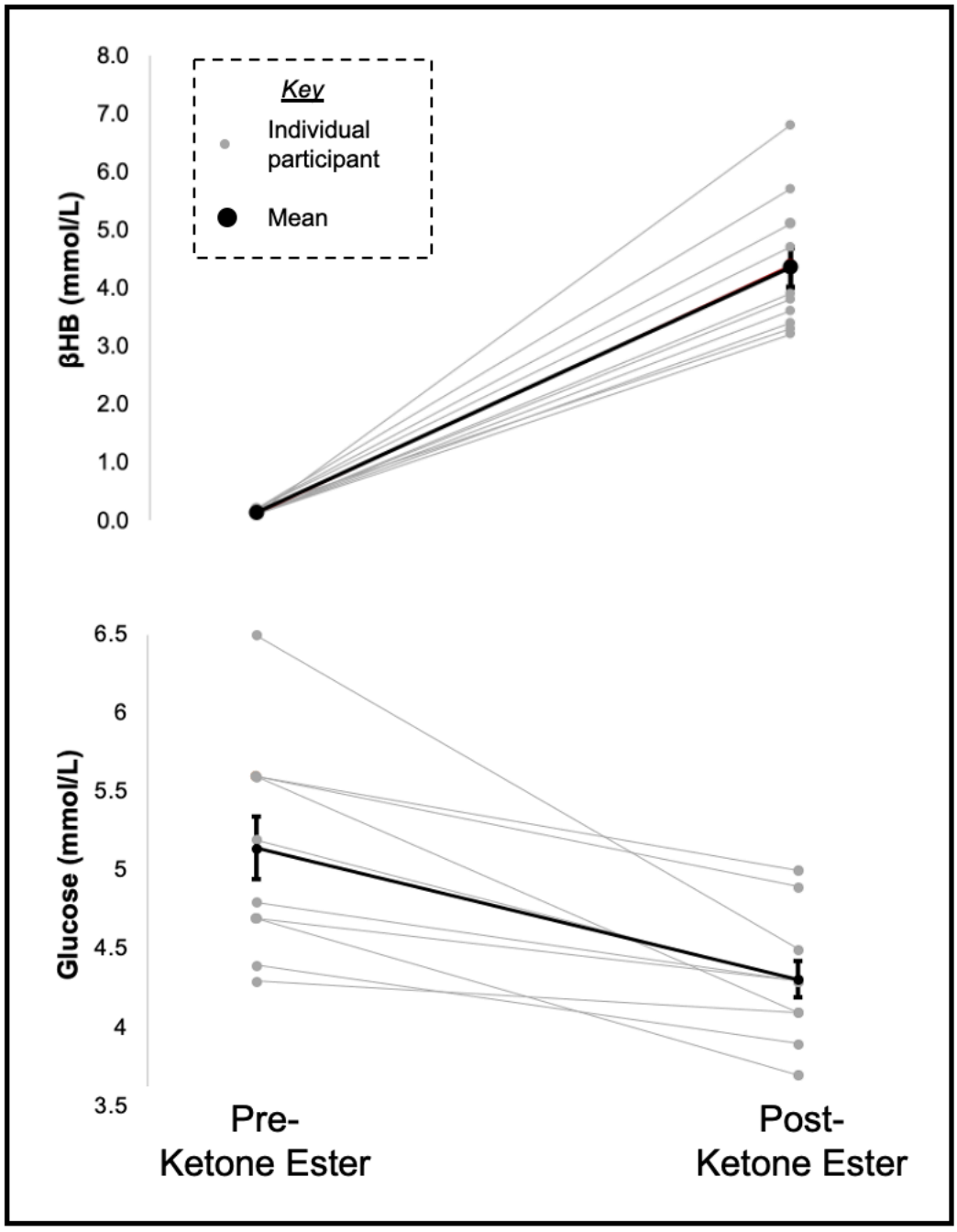


Figure 4.4 Blood βHB and glucose

Ketone ester increased βHB ($p < 0.001$) and decreased glucose ($p < 0.001$) in serum, as measured by fingerstick. Pre-ketone ester measurements correspond to directly before the 31P-MRS baseline scan and post-ketone ester measurements correspond to one hour later, before the second brain scan.

4.4.2 Ketone ester did not increase ATP level

Following absolute quantification, no change was observed in in the βATP peaks of any of the fourteen brain regions tested. The right temporoparietal cortex registered a change of 9.78 μmol/L one hour following KE consumption ($p = 0.05$), but this did not represent a significant change given the Bonferroni-corrected significance threshold of $p < 0.004$ (Table 4.1). For representative purposes, individual and mean changes in βATP for the right SN are shown in Figure 4.5.

	βATP		NAD(H)	
	MEAN DIFF (μmol/L)	P-Value	MEAN DIFF (μmol/L)	P-Value
Temporoparietal Cortex (R)	9.78	0.05	0.43	0.97
Temporoparietal Cortex (L)	0.94	0.91	4.97	0.61
Substantia Nigra (R)	1.75	0.74	-8.89	0.05
Substantia Nigra (L)	4.51	0.49	-7.27	0.17
Thalamus (R)	0.48	0.94	-9.28	0.13
Thalamus (L)	2.30	0.67	-4.62	0.43
Lentiform Nuclei (R)	-0.85	0.88	-20.1	0.04
Lentiform Nuclei (L)	0.16	0.98	-6.22	0.56
Caudate (R)	-1.72	0.79	-4.10	0.42
Caudate (L)	-1.93	0.72	-4.98	0.23
Occiptal Cortex	1.55	0.80	4.00	0.64
Cingulate Cortex	-0.40	0.95	4.03	0.18
Prefrontal Cortex (R)	1.67	0.60	9.00	0.03
Prefrontal Cortex (L)	1.91	0.59	-2.35	0.7
Bonferroni corrected p-value = 0.004				

Table 4.1 Post- minus Pre-ketone ester βATP and NAD(H) by brain region

No significant changes in either βATP or NAD(H), nor any other metabolite, occurred in any of the brain regions tested following ketone ester consumption.

4.4.3 Ketone ester did not alter NAD(H) levels

Following absolute quantification, no change was observed in in the NAD(H) peaks of any of the fourteen brain regions tested. The right SN, right lentiform nuclei, and right prefrontal cortex registered changes of -8.89 μmol/L ($p = 0.05$), -20.1 μmol/L ($p = 0.04$), and 9.00 μmol/L ($p = 0.03$) (Table 4.1); however, these did not represent a significant change given the Bonferroni-corrected significance threshold of $p < 0.004$ (Table 4.1). For representative purposes, individual and mean changes in NAD(H) for the right SN are shown in Figure 4.5.

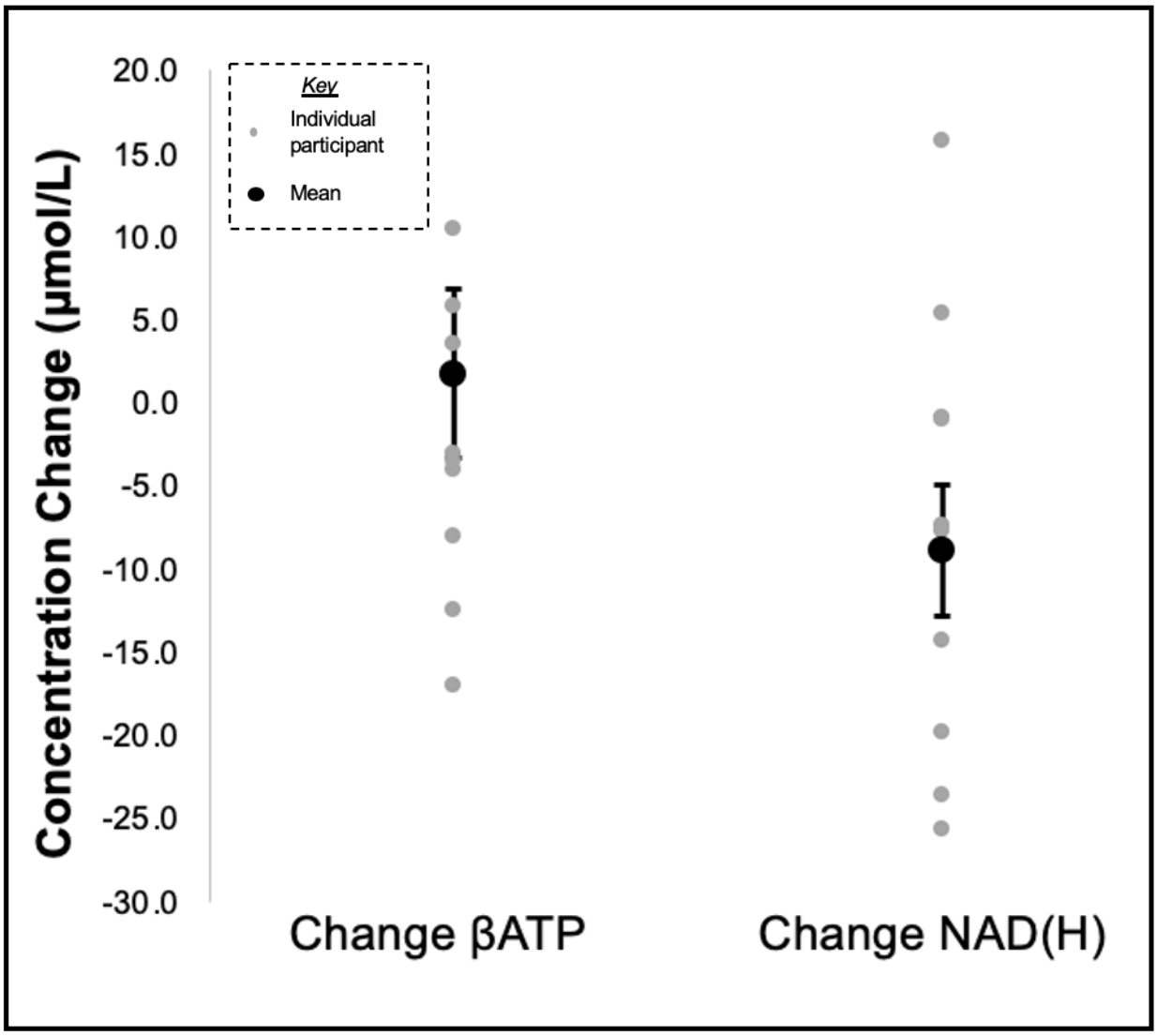


Figure 4.5 βATP and NAD(H) changes in the right substantia nigra

Participant individual and mean βATP or NAD(H) changes, one hour post-ketone ester minus pre-ketone ester concentrations are shown for the right substantia nigra as a representative example. Mean changes with standard error were 1.75 ± 5.05 µmol/L and 8.89 ± 3.91 µmol/L, respectively.

4.4.4 Ketone ester did not alter any other cerebral metabolites

No brain region registered any significant change in any other metabolites measured, including PME, Pi, GPE, GPC, PCr, αATP, or γATP.

4.5 Discussion

4.5.1 Ketone ester supplementation did not change cerebral levels of ATP or NAD(H)

Contrary to the initial hypothesis, KE did not increase ATP in any regions tested. As previous studies in PD model systems have shown βHB can increase ATP levels [81], it is conceivable that this study was simply not optimized to observe an increase in ATP. It remains possible that (i) ATP levels rose at time point different than one hour post drink, (ii) a higher dose of KE could increase ATP levels, (iii) patients with more advanced disease and more impaired cerebral glucose metabolism could benefit where early stage patients did not, or (iv) physiological adaptation to ketosis is required for persons with PD to exhibit an increase in ATP following exposure to KE. Finally, it is possible, if not likely, that (v) the pathology of PD disease is heterogenous among patients and that only specific yet-to-be classified biological subtypes of PD (perhaps those classified by the most severe complex I impairment) would exhibit an acute increase in ATP production in response to KE. For these reasons, it remains plausible that ketosis and KE supplementation could acutely increase ATP production in PD, although no evidence to support this claim was observed in this study.

The only non-ATP metabolite that reached $p < 0.05$ was NAD(H). This occurred specifically in the right SN and lentiform nuclei, a region that includes parts of the striatum to which SN dopaminergic neurons project. Despite the fact that neither change approached the Bonferroni-corrected significance threshold of 0.004, it is ostensibly compelling that the only two regions to show a decrease in NAD(H) were regions of the brain specifically impacted by PD. However, further consideration of the change suggests that it is specious. First, the spatial resolution of the 3T scan was not sufficient to completely differentiate these two adjacent brain regions. Thus,

measurements in one region were likely to correlate with the other. Second, many participants were Hoehn and Yahr stage 2 with bilateral involvement, yet the left hemisphere did not exhibit comparable shifts in NAD(H). Third, the NAD(H) spectral peak rests within the shoulder of the larger αATP peak, as can be visualized in Figure 4.3. Thus, even a small change in the αATP peak could contribute to a large skew in NAD(H) measurements. Finally, it remains unlikely, though not impossible, that a single dose of KE would be sufficient to alter NAD(H) synthesis or degradation in the timeframe of one hour. Therefore, this study reported no change in NAD(H) levels.

4.5.2 Hypothetical benefits of chronic ketone ester consumption

The ATP deficit observed in the PD brain, as compared to age-matched controls [36], is likely the result of progressive damage to mitochondria and neurons that occurs over the course of year to decades. While a DPhil thesis cannot be conducted over a sufficient timeframe to assess the neuroprotective potential of KE based on a long-term prospective interventional study in humans, the negative results presented in this chapter in no way preclude the possibility that ketogenic interventions to raise βHB levels could protect neurons and their mitochondria from further damage, ATP depletion, and other progressive metabolic upset. Therefore, this sub-section includes speculation on some of the possible long-term benefits of ketosis, as they related to PD brain energetics.

1.1.1 4.5.2a Reduced mitochondrial damage

Mitochondria are responsible for generating ATP; however, the process of ATP synthesis necessarily generates reactive oxygen species (ROS) as biproducts. When the production of ROS outweighs antioxidant defense systems, oxidative stress within mitochondria damages the electron transport chain and the mitochondrial DNA that codes for many of the proteins present within the complexes, particularly complex I. By decreasing the production of ROS and by increasing antioxidant defenses, βHB could protect the respiratory chain from accumulating damage and progressively losing its ability to sustain neurons' energy requirements.

Compared to glucose, the catabolism of βHB is predicted to generate less ROS by reverse electron transport, the main process by which ROS are produced during oxidative metabolism. In reverse electron transport, electrons are transferred back from QH_2 at complex I and leak from the respiratory chain onto oxygen to generate superoxide. βHB increases the Q/QH_2 ratio, relative to glucose, thereby decreasing a driving force for reverse electron transport [80, 100].

βHB has also been reported to decrease the mitochondrial transmembrane potential, another driving force for the generation of ROS [80, 100]. One interesting mechanism by which it may do so involves the upregulation of uncoupling protein 4 (UCP4), a brain specific UCP that dissipates excess membrane potential and diminishes reverse electron transport at complex I. Three reasons the UCP4 mechanism is particularly interesting, and possibly relevant to PD, are that (i) it directly interacts with and increases the activity of complex II [101], (ii) is highly expressed in the SN [102, 103], and (iii) modulates the pacemaking activity of dopaminergic neurons [104].

Kashiwaya and colleagues found that rats fed KE for two weeks exhibited 50% increased UCP4 expression as compared to control groups. Interestingly, no change in ATP levels were observed [105]. The following hypothesis attempts to reconcile inconsistencies in the literature that report KE increase the ΔG of ATP production in the working rat heart but does not increase ATP in the brains of Kashiwaya's rat model [80, 105]: KE increases the respiratory chain redox span and increases complex II flux, but this does not increase ATP production in the brain because it is offset by the increase in UCP4 expression. This could be adaptive by decreasing the driving force to generate ROS without sacrificing ATP generation capacity. In other words, UCP4 may finetune oxidative phosphorylation to optimize the balance between ROS and ATP. See Figure 4.6 and its caption for further details.

Thus, theoretically, chronic ketosis, as with routine consumption of KE, could permit the cleaner production of ATP and, thereby, spare dopaminergic neurons from incurring further metabolic damage. Additionally, it is important to note that the KE used in the Kashiwaya rat study was the same ΔG® KE that given to the subjects of this study and that it induced comparable increases in βHB (mean = 3.5 mmol/L) to those observed in the subjects of this study (mean = 4.2 mmol/L) [105]. Admittedly, the above hypothesis, while interesting, represents speculation that would be difficult to test in persons with PD given current technology.

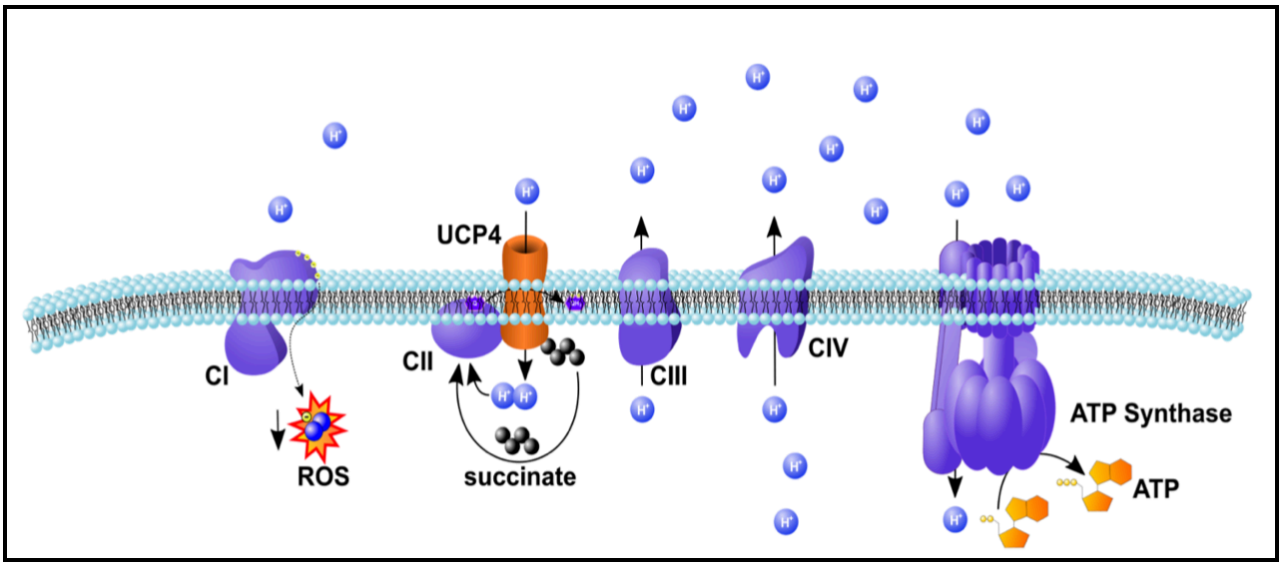


Figure 4.6 Ketone ester could upregulate UCP4 to optimize ROS-ATP balance

Mitochondrial membrane potential creates a driving force for the leakage of electrons at complex I that generates ROS. By dissipating steady state membrane potential, UCP4 decreases the production of ROS by reverse electron transport at complex I. UCP4 interacts specifically with complex II. In part by creating a local concentration of matrix protons, two of which are required to reduce each equivalent of Q, and possibly by other mechanisms, UCP4 increases succinate oxidation and complex II activity. During periods of high ATP demand, membrane potential builds and preferentially shunts electrons through the most thermodynamically favorable path into the matrix, ATP synthase. In these ways, UCP4 fine tunes oxidative phosphorylation to optimize the balance between ROS and ATP. This model explains how UCP4 decreases ROS generation to protect neurons without sacrificing ATP production capacity.

Not only can βHB decrease ROS production, but it may also support antioxidant defenses by supporting the NADPH system. This topic is later covered in chapter 7 (section 7.1.5) but is also reviewed here: βHB increases NADPH levels by decreasing glycolytic flux such that glucose-6-phosphate diverges down the pentose phosphate pathway, leading to the production of two equivalents of NADPH [80, 106-108]. βHB can further promote NADPH synthesis by increasing the concentration of mitochondrial acetyl-CoA [80] to increase the export of mitochondrial citrate by the citrate-pyruvate and citrate-isocitrate cycles, each of which generates NADPH [106, 109]. As all known intracellular antioxidants depend upon NADPH as the electron donor [109], βHB may not only decrease ROS production by reverse electron transport but may also bolster antioxidant defenses.

The net effect of decreased ROS production and increased antioxidant defenses would be less damage to the respiratory chain complexes and mitochondrial DNA and, thus, a slowing or halting of the progressive metabolic decline that occurs in PD neurons. KE may not have increased ATP levels in this ^{31}P-MRS study, but this does not exclude the possibility that chronic KE consumption is neuroprotective.

1.1.2 4.5.2b Increased cytosolic NAD^+/NADH

NAD^+ deficiency has been directly associated with brain aging and neurodegenerative disorders, and interventions that increase NAD^+ have been shown to improve age-related disease pathologies and symptoms in animal models [91, 92, 110]. For example, in rodent models of Alzheimer's disease, administration of the NAD^+ precursors, nicotinamide mononucleotide or nicotinamide riboside, have been shown to rescue mitochondrial morphology and function [111], prevent neuron cell death [112], and sustained cognitive function [112-114]. And, in a PD mouse model, a pharmacologic, P7C3, that increases NAD^+ synthesis has been shown to rescue SN dopaminergic neurons [115, 116].

The neuroprotective potential of NAD^+ derives from its role as an essential cofactor for many important longevity and cellular maintenance proteins, such as the sirtuin family of proteins and the DNA-repair enzyme, PARP1 [93-95]. The activity of these NAD^+-dependent proteins, by positively influencing oxidative stress, inflammation, autophagy, mitochondrial biogenesis, stem cell and telomere maintenance, hormonal signaling, and genomic stability, are generally thought to attenuate age-related cellular processes and neurodegeneration. Beyond regulating sirtuins and PARP1, NAD^+ is considered one of the "great controlling nucleotide coenzymes," compounds that chiefly govern metabolism and, by extension, cellular function [109].

If NAD^+ deficiency contributes to neurodegeneration, and NAD^+ boosting treatments could play a role in modifying disease, the next question to ask would be whether ketosis and βHB could positively influence NAD^+ metabolism. There are mechanistic reasons to believe the answer is yes. βHB catabolism spares cytoplasmic NAD^+ that would otherwise be consumed during

glycolysis. βHB can also decrease oxidative stress and DNA-damage, alleviating competition between PARP1 and sirtuins for NAD^+. Ketosis also influences NAD^+ biosynthesis and salvage pathways. For example, ketogenic diets alter the activity within the kynurenine pathway, which makes NAD^+ from tryptophan [117]. βHB also activates AMPK, which upregulates the rate-limiting enzyme in NAD^+ salvage, NAMPT [118, 119]. Though the universe mechanisms have not been thoroughly explored, the point remains that there is large overlap between βHB and NAD^+ metabolism.

Preclinical and clinical studies also suggest ketogenic interventions could benefit NAD^+ metabolism. The Kashiwaya study referenced above, in which rats were administered KE for two weeks, observed an increase in cytoplasmic NAD^+/NADH [105]. Similar positive findings have been observed in rodents on ketogenic diets, corresponding with increased activity of NAD^+-dependent sirtuin proteins [120, 121]. Functionally, a ketogenic diet and βHB each improved phenotypes in models of Cockayne syndrome, a neurodegenerative disorder characterized by NAD^+ depletion [122]. And, in young healthy humans, Xin and colleagues found that medium chain triglyceride supplementation to induce low-level ketosis also appeared to increase the NAD^+/NADH ratio [99].

Thus, methodological logic, preclinical studies, and one human study suggest an interesting intersection between βHB and NAD^+/NADH. Future studies that aim to explore the disease-modifying potential of ketogenic interventions for neurodegenerative and neurological diseases should include investigations of NAD^+/NADH metabolism.

4.6 Summary

Parkinson's disease and other neurodegenerative disorders are marked by an energy deficient brain. This study investigated whether a single dose of KE could increase ATP levels in the brains of 11 patients with PD, as measured by ^{31}P-MRS. No change in ATP, nor in any other metabolite measured, was detected one hour after KE consumption. While these data do not provide evidence that exogenous βHB acutely increases ATP levels in PD, neither do they contradict the possibility that chronic ketosis could be neuroprotective. Other, mostly preclinical, evidence suggests that ketosis and βHB can reduce mitochondrial damage and improve NAD^+ levels to prevent further impairments in energy metabolism and disease progression. Clinical studies are required to properly assess the impact of long-term ketosis on energy metabolism in the brain.

Chapter 5. *28-day study pilot: Feasibility of regular ingestion of ketone ester and validation of methods*

5.1 Abstract

Human clinical trials have demonstrated that ketogenic diets are effective in addressing Parkinson's disease symptoms. This randomized placebo-controlled study was designed to investigate whether exogenous ketosis, induced by ketone ester and separate from carbohydrate restriction, could also be useful in treating Parkinson's disease. It employs a broad array of motor, non-motor, and other tests in order to capture the potential effects of ketone ester consumption on patients' symptoms. Study setup, including design, ethics, and contracts, is complete. Additionally, four participants completed the intervention phase of the study before the COVID-19 pandemic forced study suspension. Therefore, this chapter is included as a feasibility and validation study.

5.2 Background

5.2.1 Ketogenic diet in Parkinson's disease

The first study to investigate the potential benefits of ketosis in Parkinson's disease (PD) occurred in 2005. In this small pilot, seven patients were recruited to consume a ketogenic diet (90% calories from fat) for 28 days. Five participants completed the trial, but only three were able to faithfully adhere to the diet, highlighting potential compliance issues and a use case for the ΔG® ketone ester (KE). All participants who achieved ketosis for the majority of the 28 days experienced clinically meaningful improvements in symptoms as measured by Unified Parkinson's Disease Rating Scale (UPDRS) motor scores. On average, completed participants' scores improved 43%, with improvements ranging from 21% to 81% decreases on the UPDRS [37].

More recently, a trial including 38 persons with PD compared the impacts of eight-week isocaloric low-fat and ketogenic diets (78% calories from fat). While both groups exhibited improvements relative to baseline in all four categories of the Movement Disorder Society-UPDRS (MDS-UPDRS), the low-fat group outperformed the ketogenic group in no categories, whereas the ketogenic diet improved from baseline in non-motor symptoms (including fatigue and cognitive impairment) significantly more so than the low-fat group (41% versus 11% improvement, $p<0.001$) [38].

5.2.2 Exogenous ketone supplementation in Parkinson's disease models

While the mechanisms by which ketosis may improve the symptoms of PD in humans are unknown, *in vitro* and animal work suggests βHB might be able to increase energy production or dopamine synthesis in the PD brain. For example, Tieu and colleagues concisely demonstrated that administration of βHB increased ATP and protected against decline in dopamine levels in an *in vitro* model of PD and protected motor performance in a mouse model of PD [81]. (For more on this topic, please refer to chapter 7.) Admittedly, the hypothesis that βHB increases ATP production acutely is not supported by the chapter 4 ^{31}P-MRS data, which showed no increase in ATP one hour following the administration of KE.

5.2.3 Symptomatic management versus disease-modifying adjunctive therapy

It is worth reinforcing the point that PD does not lack effective symptomatic treatments, but disease-modifying treatments. While levodopa works to manage patients' symptoms, at least for a limited period of time, PD inevitably progresses as dopaminergic neurons die. Considering the disease-modifying and neuroprotective mechanisms of ketosis highlighted in chapter 7, the true potential for ketogenics, as relates to PD, may not be as a symptomatic treatment but as an adjunct to symptomatic treatments that prevents the disease from progressing.

As it would not be feasible to assess whether ketosis slows the clincal progression of PD in humans within the duration of a DPhil thesis, none of the three clinical studies presented in chapters 3 – 5 were designed to directly investigate the disease-modification question. Nevertheless, it is worth clarifying the distinction between symptomatic management and protection against disease progression.

5.3 Study overview

This study was designed to involve 20 participants randomized to consume either KE (25 mL dose) or taste-matched placebo control drinks (25 mL water with Symrise bitter flavor, product code: SY648352) four times daily for 28 days. The desired sample size was determined using the methodology given in section 2.5.2 (and based on the UPDRS improvements previously observed in patients on ketogenic diets for 28 days [37]). Participants were randomized using the block-four protocol given in section 2.5.3.

Although the intervention itself spans 28 days, each participant will be followed for 14 days prior to and following the intervention in order to establish baselines for comparisons and investigate possible lasting or lagging effects of the intervention. Thus, the total duration for which participants will be recruited is two months (Figure 5.1). During these two months participants will be assessed on-site at the John Radcliffe hospital and will also be required to engage in at home tests. The details of these tests are the subject of the next section.

5.4 Participant assessments

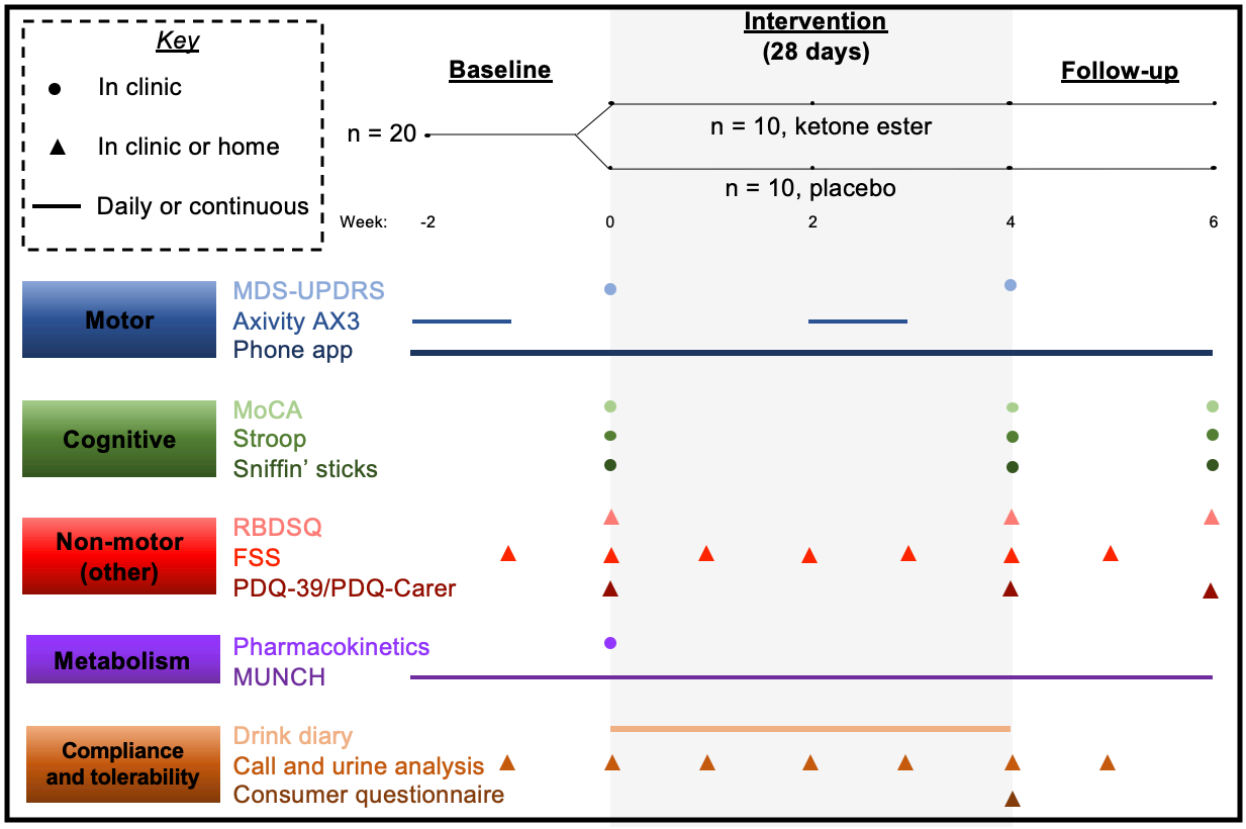


Figure 5.1 28-day study schematic

Twenty participants will be randomized to ketone ester and placebo groups. After a two-week baseline period, participants in each group will consume their respective study drinks four times daily for 28 days (grey shaded area), prior to a two-week follow-up. Participants will be seen in clinic fortnightly, as well as be monitored remotely. The tests can be clustered into those assessing motor function, cognition, non-motor symptoms other than cognition, metabolism, and compliance with the protocol and tolerability of the study drink. The circles, arrowheads, and bars indicate when each test will be administered. Abbreviations: MDS-UPDRS, Movement Disorder Society-Unified Parkinson's Disease Rating Scale; MoCA, Montreal Cognitive Assessment; RBDSQ, rapid eye movement sleep behavior disorder screening questionnaire; FSS, fatigue severity survey; PDQ-39, Parkinson's disease questionnaire-39; PDQ-Carer, Parkinson's disease questionnaire for carers; MUNCH, My Ultimate Nutrition Calculator and Helper.

5.4.1 Motor tests

5.4.1a MDS-UPDRS

The MDS-UPDRS is the standard clinical assessment of PD symptom severity. It consists of four parts:

(i) Part I assesses non-motor symptoms by questionnaire.

(ii) Part II assesses motor symptoms by questionnaire.

(iii) Part III assesses motor symptoms by physical examination.

(iv) Part IV assesses motor complications associated with the prolonged use of levodopa, such as dyskinesias (random involuntary jerky movements), motor fluctuations (when patients do not respond to levodopa), and dystonia (painful cramping).

Participants will be assessed at the beginning and end of the 28-day intervention (Figure 5.1).

5.4.1b Axivity AX3 continuous monitoring

Participants will wear a continuous activity monitor, the Axivity AX3, for one week during the pre-intervention baseline and for one week during the intervention. The inclusion of continuous activity monitoring provides the prospect of collecting higher powered data with the potential to reveal impacts of the KE intervention on motor function that might otherwise be lost in the noise of intraperson variability on the MDS-UPDRS. This notion is supported by existing data:

Cavanaugh and colleagues found that the total number of daily steps and the intensity of activity (such as “intensity minutes”: the number of minutes in a day that persons with PD record 100 steps per minute) declines overtime. In their study of 33 persons with PD, number of steps and intensity

minutes decreased by 12% and 40%, respectively, over one year ($p<0.01$). Importantly, these declines were not associated with declines in UPDRS scores because the UPDRS is more susceptible to symptom variability [123].

While multiple monitor options were available for continuous activity monitoring, the Axivity AX3 (Figure 5.2) was chosen because it is well-suited for participant comfort and enables further collaborations with the Oxford Brookes University team who assisted in conducting the endurance exercise study (chapter 3), are able to provide a supply of AX3 monitors, and possess the software and expertise to analyse the raw data.

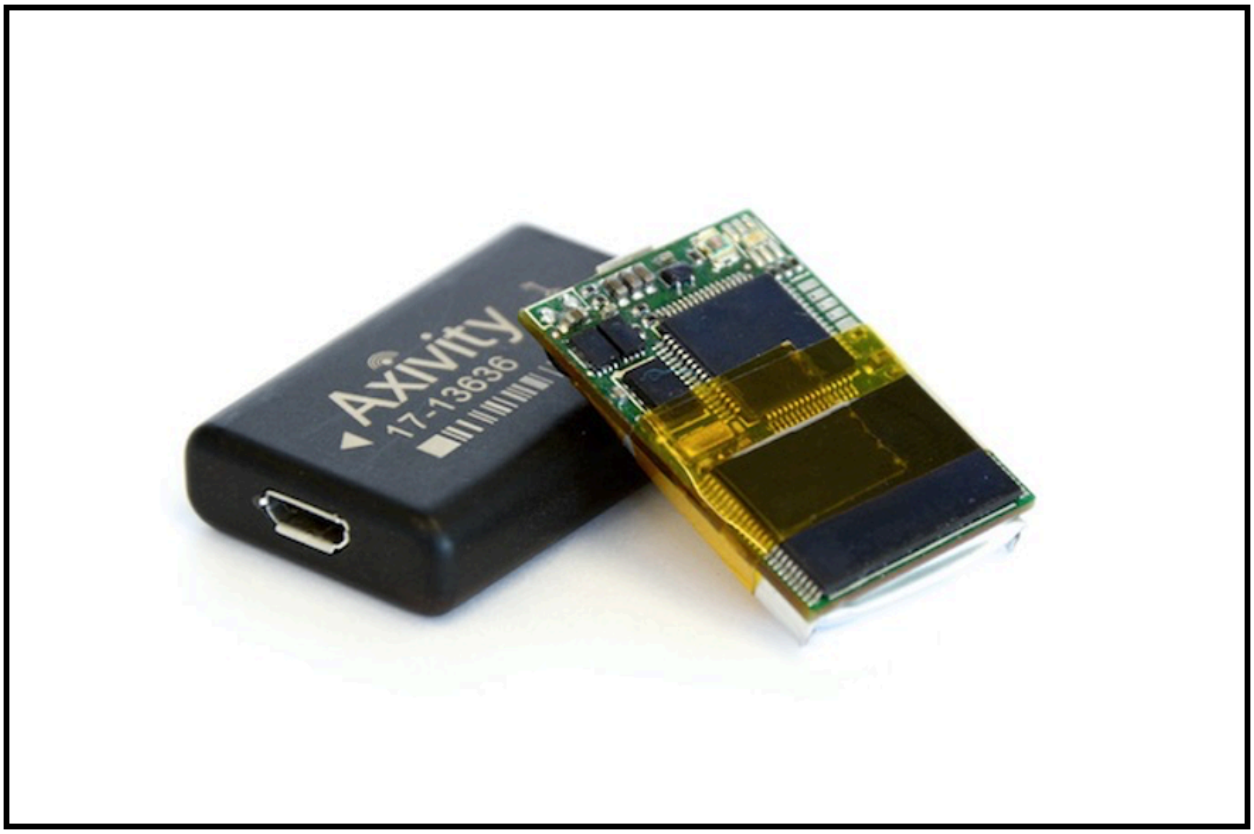


Figure 5.2 Axivity AX3

The Axivity AX3 is an 11 gram 5.7 cm^3 water-resistant accelerometer that participants wear continuously for seven days each during the baseline and interventions periods to assess the impact of study drink consumption on daily activity. (Image from the Axivity user manual, https://axivity.com/userguides/ax3/.)

It was determined by consensus that the best locations to attach the monitors to participants would be either the lower back (above the fifth lumbar vertebrae) or to the wrist of the arm less afflicted by tremor. Axivity AX3 use has been validated for both sites in persons with PD [124, 125]. To determine which location would better serve the purposes of the study, one Axivity AX3 was applied to each site on the first four participants. As participants found that the monitors attached to the back were less comfortable and fell off more easily, monitors will be attached to future participants using wrist strap only.

For data collection, the monitors were and should be set to collect at a frequency of 100 Hz and with a range of 8 g-force. These parameters are compatible with the software that the Brookes team will use to perform the raw data analysis, retains the power to capture rates of acceleration persons with PD are likely to exhibit, and maintains a two-week battery life.

Participants will wear the monitors for 14 days total from weeks -2 to -1, as a baseline, and from weeks 2 to 3, during the intervention (Figure 5.1).

5.4.1c Smartphone application

The Oxford Parkinson's Disease Center (OPDC) team recently developed a smartphone application that allows patients to assess symptomology independently at home. It has been clinically validated and, in a study of 237 persons with PD that coupled the raw data with a machine learning algorithm, was shown to accurately predict future clinical outcomes over an 18-month follow-up [126].

The inclusion of the smartphone application, similar to the Axivity monitor, provides an opportunity to collect high powered data on motor symptoms. Furthermore, as it has been shown to predict clinical progression, the data it generates could be used to perform subgroup analyses on data generated by other tests included in this study.

The application itself involves seven distinct tests that take a collective seven minutes to complete. The tests are summarized as follows:

(i) Voice: Participants say "ahh," as a steady breath, for 20 seconds or as long as possible.

(ii) Balance: Participants put the phone in their pockets and stand still for 20 seconds.

(iii) Gait: Participants keep the phone in their pockets and walk across the room and back.

(iv) Dexterity: Participants use their index and middle fingers to tap buttons that appear on the screen (Figure 5.3).

(v) Reaction time: Participants press a yellow button as fast as possible when it appears on the screen and hold it down until it disappears.

(vi) Resting tremor: Participants rest their hands on a flat surface and hold the phone as steady possible.

(vii) Postural tremor: Participants hold the phone in their outstretched hands as steady possible.

Participants were and should be instructed to complete all seven tests three times daily and at the same time each day for the entire two months that they were or will be involved in the study.

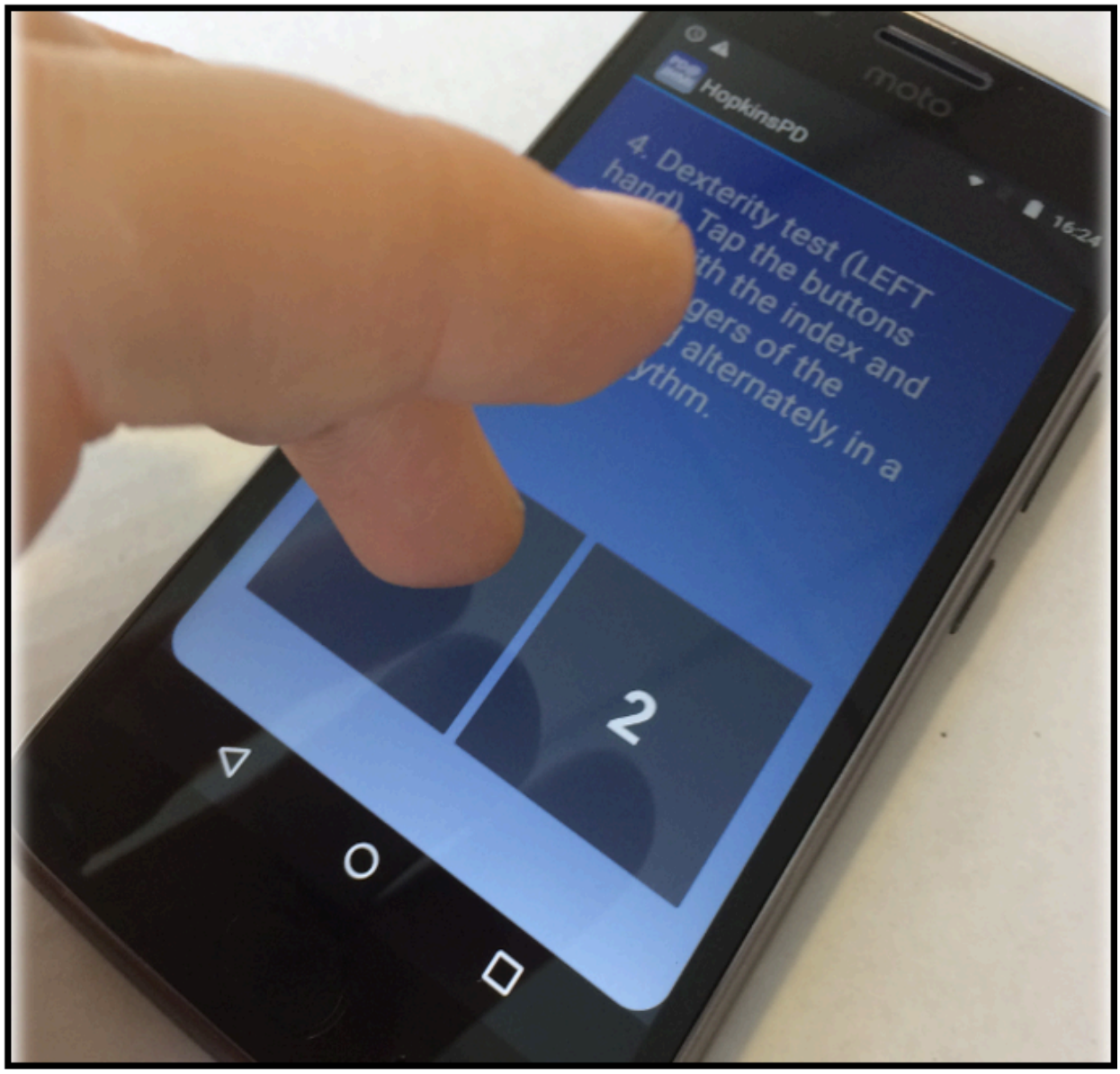


Figure 5.3 Smartphone application

The Oxford Parkinson's Disease Center (OPDC) developed and validated a smartphone application that participants use three times daily to assess motor symptoms. Above is shown the dexterity test, which is the fourth of seven such at-home tests. (Image from the OPDC user guide.)

5.4.2 Cognitive tests

5.4.2a MoCA

The Montreal Cognitive Assessment (MoCA) is a standard neurocognitive test for cognitive impairments composed of a one-page written assessment and verbal tasks that evaluate memory, attention, language, and visuospatial skills. The MoCA was selected as a cognitive assessment tool because it has been documented to be more sensitive for detecting cognitive impairments in PD as compared to other standard assessments, such as the Mini-Mental State Examination [127].

5.4.2b Stroop

The Stroop color-word test evaluates selective attention and processing speed. The test involves reading two lists and saying aloud the font color of written color names, such as “red” and “blue.” The first list is printed such that the color names are congruent with the colors in which the names are printed. For example, “blue” is printed in blue font. The second list is printed such that the color names are incongruent with the colors in which the names are printed. For example, “blue” is printed in red font. This creates an interference effect that forces participants to attend to the color itself and ignore the identity of the word (Figure 5.4).

Stroop performance is impaired in persons with PD as compared to healthy controls, both with respect to how long it takes them to complete the task and how many errors they make [128-130]. Participants’ individual performances on the noncongruent test were and will be compared to their performances on congruent test as a measure of interference. If KE improves selective attention capacity, this may be detected as a decrease in the time it takes participants to complete the noncongruent test.

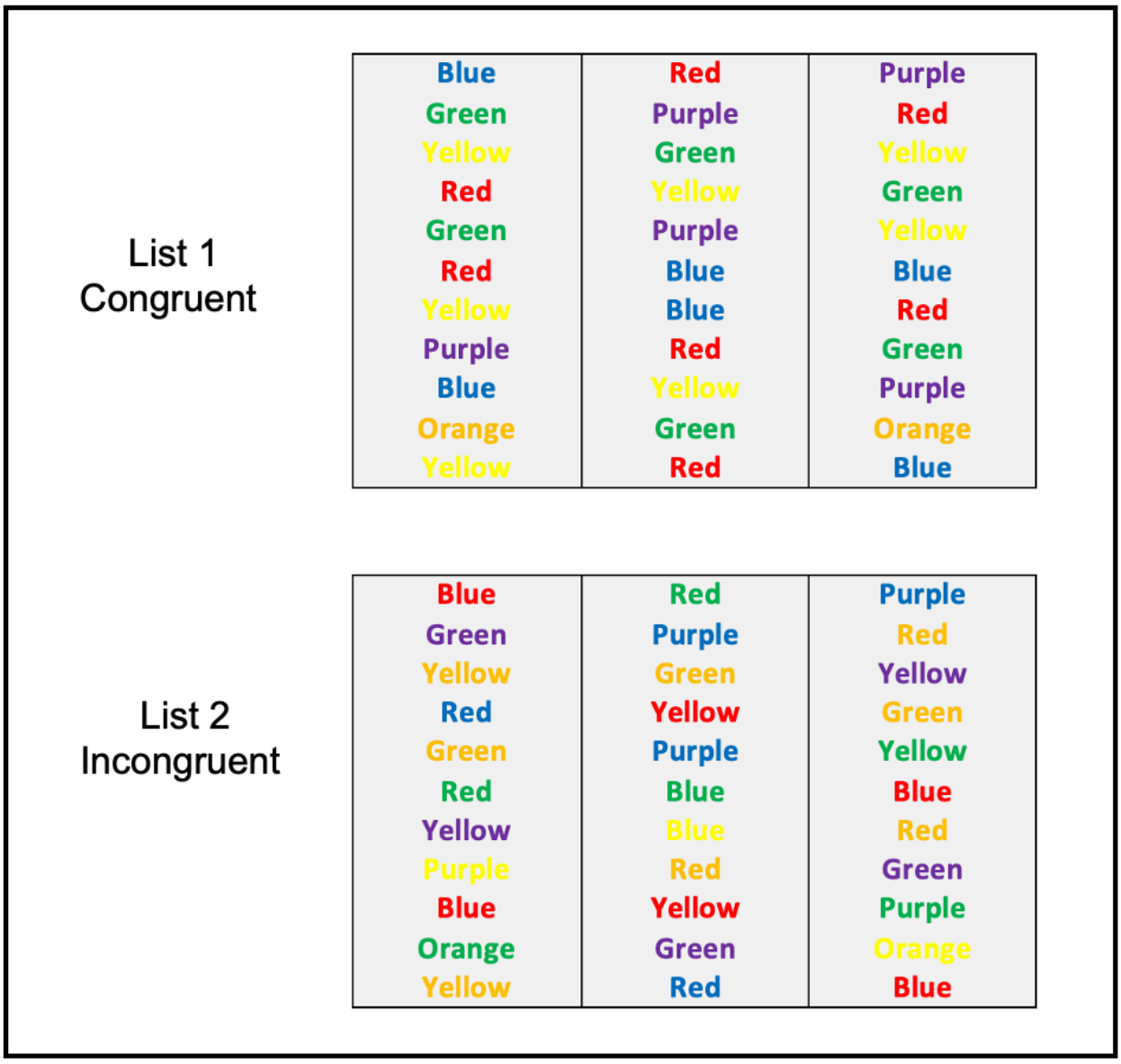


Figure 5.4 Stroop test

The Stroop color-word test assesses selective attention and processing speed. Participants first read the colors on list 1, in which colors and words are congruent, followed by reading the colors on list 2, in which colors and words are incongruent. Timed performance on list 2, ratioed to performance on list 1, is measured as the interference effect.

5.4.2c Sniffin' sticks

Hyposmia is a common prodromal symptom in PD. While there are no studies or published case reports documenting recovery of smell in PD after a prolonged period of ketosis, it has been anecdotally reported that exposure to ketosis is associated with improvements in olfaction.

The possibility that ketosis could contribute to even partial resolution of PD-associated hyposmia is intriguing given that a main center for neurogenesis in the adult brain is the ventral tegmental area, which sends new neurons to the olfactory bulb and olfactory network [131, 132]. Furthermore, studies in model systems have found that βHB induces the expression of brain-derived neurotrophic factor (BDNF) [47, 85], a nerve growth factor that is depleted in PD [133] and that has also been shown to support the survival of newborn olfactory neurons and sense of smell in rodents [134].

Therefore, it is possible that KE supplementation could contribute to some level of neurogenesis or neuroprotection in the brains of patients with PD and that this might be functionally detected as a recovery of smell, as has been anecdotally reported. While this possibly may be unlikely in the timeframe of the study, given that the half-life of olfactory neurons is on the order of 90 days [135], the Sniffin' sticks tests provided a quick and cheap way to investigate the question.

All three cognitive tests were and will be administered to participants both at baseline and at the end of the intervention (Figure 5.1).

5.4.3 Other non-motor symptoms

5.4.3a RBDSQ

In addition to hyposmia, rapid eye movement (REM) sleep behavior disorder (RBD) is common in PD and often manifest years before motor symptom onset, in association with loss of dopaminergic neurotransmission [136, 137]. Furthermore, presence of RBD in PD, as compared to PD absent of RBD, is associated with worse non-motor symptoms and quality of life, different frequency of tremor, increased likelihood of demonstrating hyposmia, and altered responsivity to medications [138-140]. This has led to the hypothesis that the presence or absence of RBD might reflect different pathological subtypes of PD.

The RBD screening questionnaire (RBDSQ) is designed to evaluate the presence of RBD and exhibits 84% sensitivity and 96% specificity as compared to polysomnography [141]. The RBDSQ was included in this study for two purposes: First, to investigate the possibility that KE supplementation affects sleep quality in PD; and, second, to stratify participants following data collection so that subgroup analyses could be performed that might reveal responsivity to the intervention among potentially pathologically distinct groups of patients.

It was determined that a score of 5 or fewer out of 13 points on the questionnaire would be used as a cut-off for diagnosing probable RBD based on prior data showing that an RBDSQ threshold of 5 diagnoses probable RBD in 47% of PD cases and polysomnography diagnoses RBD in 46% of PD cases [138, 142].

The RDBSQ was and will be administered at the beginning and end of the intervention and at follow-up. It is recommended that participants involve their bed partners in filling out the form, as bed partners may have insight in participants' sleeping behaviors to which participants themselves do not. Therefore, when applicable, participants were and should be encouraged to take the questionnaire home, fill it out with input from their bed partners, and return it at the next scheduled appointment (Figure 5.1).

5.4.3b Fatigue Severity Survey

Fatigue is a common symptom in PD that negatively impacts quality of life. The fatigue severity survey (FSS) is nine question survey that has been validated in PD [143]. The FSS was and will be administered over the phone during weekly compliance checks (section 5.4.5b, below) in order to assess fatigue weekly and under free-living conditions, rather than fortnightly under laboratory conditions and when participants are fasted (Figure 5.1).

5.4.3c PDQ-39 and PDQ-Carer

Neurodegenerative diseases are family conditions that impact not just the person afflicted with the pathology but also their loved ones, particularly their care partners. Therefore, it is important to assess overall quality of life both in participants and their partners using the complementary standard PD quality of life questionnaires: the PD questionnaire-39 (PDQ-39) for participants and PD questionnaire for carers (PDQ-Carer) for care partners. The questionnaires were and will be administered at the beginning and end of the intervention and at follow-up (Figure 5.1).

5.4.4 Ketone ester metabolism

5.4.4a Pharmacokinetics

The pharmacokinetic profile of KE was previously documented by the Clarke group's Dr. Brianna Stubbs [58]. To confirm that this metabolic profile is not altered in PD, participants will be offered the option to opt in to an additional single blood test in which they come into the lab fasted, consume a standard dose of the study drink, and have small 1 - 2.5 mL blood samples taken from an IV cannula at 0, 10, 20, 30, 60, 90, 120, 150, 180, and 240 minutes post-drink. Each sample will then be measured for βHB, acetoacetate, glucose, free fatty acids, and triglycerides. The pharmacokinetic assessment was an amendment and, thus, no participants have yet completed it at the time of writing of this thesis.

5.4.4b MUNCH

My Ultimate Nutrition Calculator and Helper (MUNCH) is the nutrition tracking research tool that is the topic of chapter 8. The COVID-19 pandemic, in delaying study completion, provided an opportunity to further develop this tool for future use in this study. The application of MUNCH in a subgroup of study participants will allow the investigation of two questions: First, how does the KE drink, which is presumed to be appetite suppressing, impact participants' intakes of specific nutrients? And, second, do specific nutrients interact with the drink or independently affect symptomology?

5.4.5 Compliance and tolerability

5.4.5a Drink diary

As one metric of compliance, participants were and will be asked to record when they consumed their study drinks in a drink diary. This diary also provides the opportunity to capture other potentially useful information, including sleep duration, frequency of exercise, and mealtimes (Appendix 5).

5.4.5b Call and urine analyses

To further assess compliance, participants were and will be called once per week at a random time (Figure 5.1). They were and will be asked when they had last consumed the study drink and to immediately follow the call by urinating on an acetoacetate test strip that turns from pink to purple when participants are in ketosis (Figure 5.5).

Participants in the KE group who respond that they have not taken a dose of KE for more than four hours (and, therefore, may no longer be in ketosis), or who register as negative on the acetoacetate test strip, were and should be marked as noncompliant. As compliance in the placebo group cannot be assessed by test trip, compliance in this group is based only on participants' responses.

Participants were not and should not be provided with the key for the test strip, such that they remain blinded to their conditions.

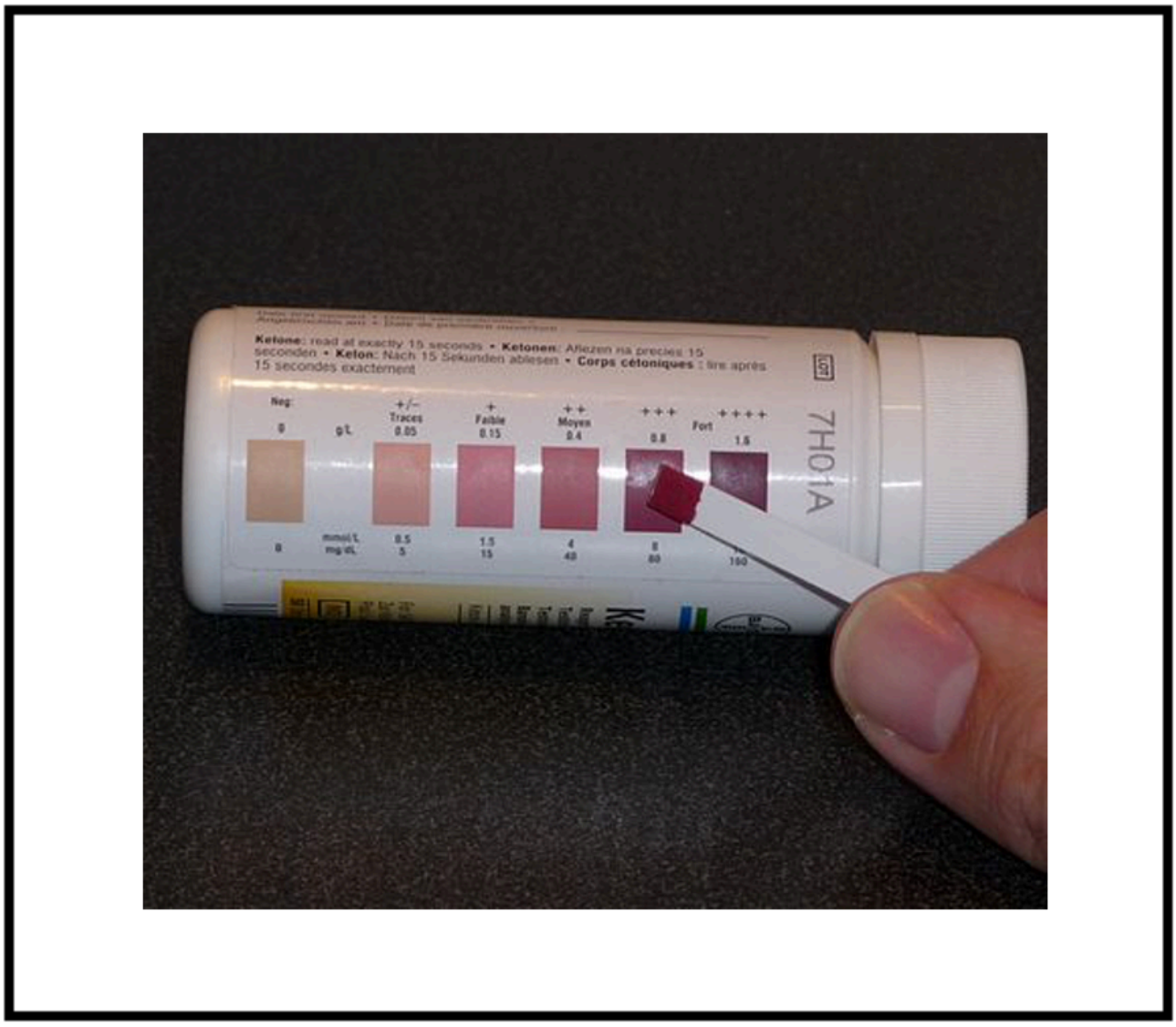


Figure 5.5 Urine strips

Urine strips determine the presence or absence of ketosis by detecting acetoacetate. Darker color indicates ketosis. (Opensource image of Bayer Ketostix testing strips.)

5.4.5c Consumer questionnaire

The KE drink is particularly bitter and unpleasant tasting, which could impair patients' compliance to the protocol as well as to a four times daily regimen of KE supplementation long-term. Therefore, it is important to assess participants' tolerability and subjective experiences with KE and whether they feel that they would be likely to use it beyond the confines of study, were it publicly available. To this end, an eight-question questionnaire was developed and administered to participants at the end of the intervention (Appendix 6).

5.5 Impact of COVID

5.5.1 Study suspension

In March 2020, after four participants had completed the intervention phase of the study, the advent of the COVID-19 pandemic and lockdown required the suspension of clinical studies, particularly those involving elderly participants or those with pre-existing metabolic conditions.

5.5.2 Future of study

After several months of lockdown and my relocation to the United States, my supervisor, research team, and I determined that it would not be feasible for me to continue as the student lead of this third and last clinical study. Instead, we determined the most responsible course of action, both for patient safety and for study integrity, would be for me to transfer the study to incoming Clarke group DPhil student, Adam Isherwood. As I was chiefly responsible for the design, setup (including ethical approvals and contracts) and completion of the first four participants, I plan to remain involved in this study by helping Adam with the analyses and with writing up the data while I am in medical school and once the study is complete for his DPhil.

Chapter 6. *My Ultimate Nutrition Calculator and Helper (MUNCH): A nutrition tracking tool for improving nutrition resarch*

Declaration

I certify that the application described in this chapter – MUNCH – represents entirely my own idea and is based on my independent work. I built a useable prototype, based on nested Microsoft excel sheets, that possesses all the functions described herein and that is available upon request. However, as I do not possess the computer coding expertise required to build web- and smartphone-based versions of my application, I contracted a professional, Izaak Prats, to convert my prototype into version 1 that can be accessed online via computer or smartphone. I financed the building of this prototype and am not conflicted by association with any industry, nor do I intend to develop this tool for personal profit. I am creating MUNCH for altruistic research and clinical purposes. I include this declaration to reinforce that I understand the University of Oxford's policy on plagiarism and that the contents of this chapter represent my ideas and that the MUNCH application is based on a useable prototype that I myself developed.

6.1 Abstract

Nutrition research that relies on food frequency questionnaires or participant recall surveys provide limited information of questionable accuracy. Studies that rely on metabolic wards or meal provisions are short-term or are otherwise prohibitively expensive. My Ultimate Nutrition Calculator and Helper (MUNCH) is a unique nutrition tracking tool to enable the conduct of inexpensive research studies that collect unprecedently broad and accurate dietary intake data in free-living populations. It is based on the concept that the researcher customizes the tool to best fit the research question at hand. MUNCH can also, in future, be deployed as a clinical tool for the management of chronic diseases. Currently, MUNCH is a web-based version 1. Additional features will be incorporated over the coming years to convert MUNCH from a proof-of-principle model into a scalable tool for widespread research and clinical use.

6.2 Background: Limitations of nutrition research

As the field of nutrition research stands, no single study can simultaneously fulfil the following three criteria:

(i) Collects accurate data

(ii) Conducted long-term (and in free-living populations)

(iii) Inexpensive

Food frequency questionnaires or patient recall surveys can be used to fulfil criteria (ii) and (iii), but they do not provide detailed or reliable data. They may ask questions like, "how often in the last month did you eat red meat?" or, "how often do you consume eggs or products containing eggs?" Such questions are fraught with confounds. How much was each portion size? What type of red meat? Is a lean venison steak equal to a fast-food hamburger? Was there sauce? How were the eggs cooked? Were they organic or pastured? Does a croissant or other pastry count as an egg-containing food? What other nutrients were consumed along with these foods?

More rigorous methodologies to accurately assess participants' intakes include metabolic ward studies or providing participants with all their meals during the course of a study. Such strategies fulfil criteria (i) but can be incredibly expensive. Thus, they can only be conducted for a short period of time or become economically infeasible for many research groups not funded by industry. Thus, research employing more accurate data collection methods often must choose between criteria (ii) and (iii).

There is currently no tool that enables the collection of accurate data in a cheap manner and over long periods of times in free-living populations. Were such a tool to exist, it would broaden the types of questions nutrition researchers could ask and answer.

6.3 Method: MUNCH as a nutrition research tool

6.3.1 Overview

My Ultimate Nutrition Calculator and Helper (MUNCH) is based on the concept that, in order to collect accurate data that are relevant to a particular research question, researchers need to be able to personalize the tool to their specific use cases. This approach is distinct from available nutrition tracking applications, which rely on pre-set parameters and large food databases that carry limited information and are often inaccurate when applied to individual end users.

On the MUNCH platform, researchers can easily build the tool themselves to better suite their project. For example, no prior nutrition tracking tool allows for the tracking of omega-6/3 ratio intake or for the intake particular micronutrients, such astaxanthin or hydroxytyrosol. These and other such nutrient variables are relevant to chronic inflammatory, neurodegenerative, and neurological diseases. Until MUNCH, there was no way to track such variables while fulfilling the above three criteria.

Thus, MUNCH has the potential to address current limitations in nutrition research and fulfil all three parameters of the accurate-long-term-inexpensive triad. The upcoming section 8.3.2 will describe the fundamental features and setup of the MUNCH platform.

6.3.2 Personilzed parameters

6.3.2a Nutrient "Goals"

The first step in building a MUNCH platform for a particular project is to define the nutrients of interest or "Goals." These can include any and all variables the researcher wants to monitor.

To add a new Goal, the researcher simply selects "Add New Goal" and then types in the name of the nutrient of interest. The researcher can also choose to define target ranges for a given goal or define only minimums or maximums. Such ranges, minimums, and maxims could, for example, correspond to Recommended Daily Allowances and Upper Tolerable Limits for particular micronutrients (Figure 6.1).

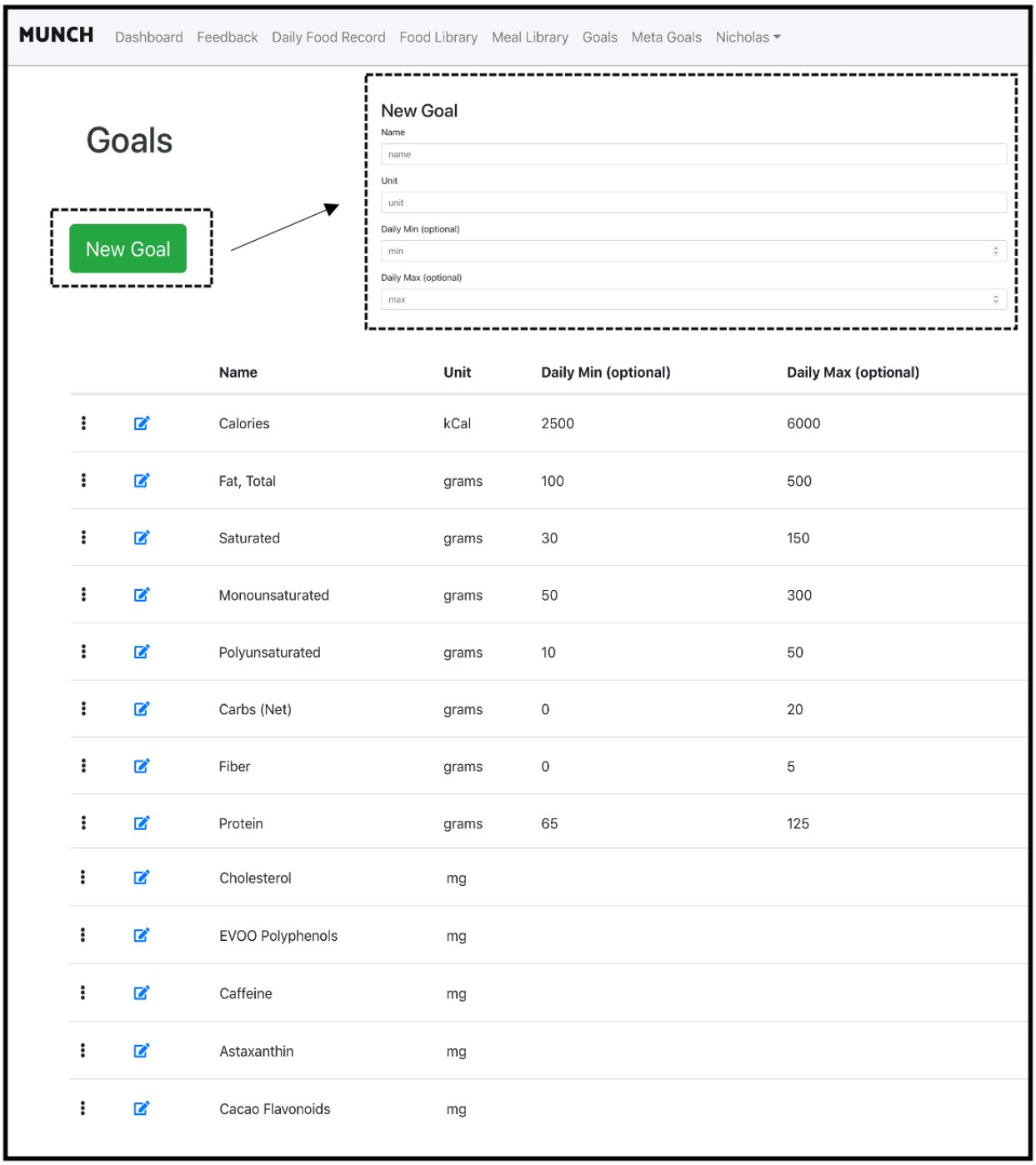


Figure 6.1 Nutrient Goals

To add a goal, researchers select “New Goal” (green button) and are then prompted to enter the parameters for that goal (inset), including units and an optional target range, minimum, or maximum.

6.3.2b Meta Goals

In addition to tracking individual nutrients, researchers can track the relationship among nutrients using "Meta Goals." Meta Goals can be either additive totals of Goals, ratios among Goals, or percentage breakdowns of a Goal. As examples, a researcher could use Meta Goals to monitor total long-chain saturated fat intake by adding the intakes of particular saturated fatty acids, omega-6/3 ratio by ratioing omega-6 intake to omega-3 intake, or percentage intake of total fat as saturated, monounsaturated, and polyunsaturated fats (Figure 6.2).

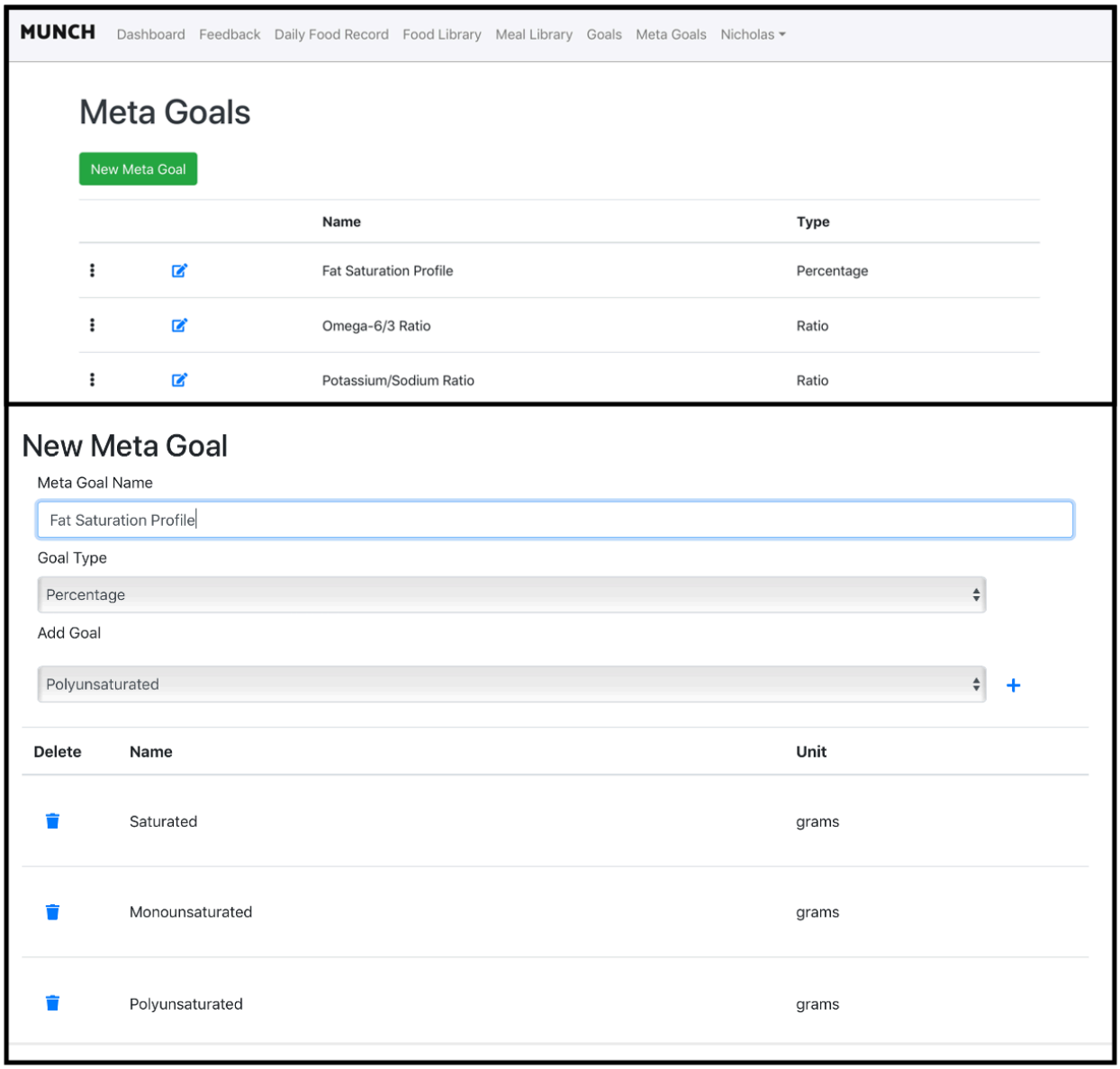


Figure 6.2 Meta Goals

Researchers can create Meta Goals that include totals, ratios, or percentages. In the above example, the researcher is creating a Meta Goal that will output the percentage of total fat intake made up of each saturated, monounsaturated, and polyunsaturated fats.

6.3.2c Food Library and units

After defining the parameters of interest, the researcher builds a "Food Library" for each participant consisting of the foods the individual consumes. When adding a "New Food," the researcher is prompted to fill in all the data for the parameters of interest (Figure 6.3). These data, corresponding to the exact food the participant eats (brands and varieties), can be sourced directly from nutrition labels and integrated with data from reliable sources, like PubMed or the United States Department of Agriculture's Food Central database.

Furthermore, and importantly, users can define the portion sizes in a manner that makes sense for participants in order to ease the tracking process for participants. For example, rather than define one unit of lettuce as "one cup," which may be confusing for participants, the unit could be set to "1 bag" of Tesco romaine lettuce. The approach of building a customizable food library for participants, based on data sourced from the specific foods participants actually eat and using units they find sensible, improves tracking accuracy and shifts the burden of measurement from the participant to the researcher.

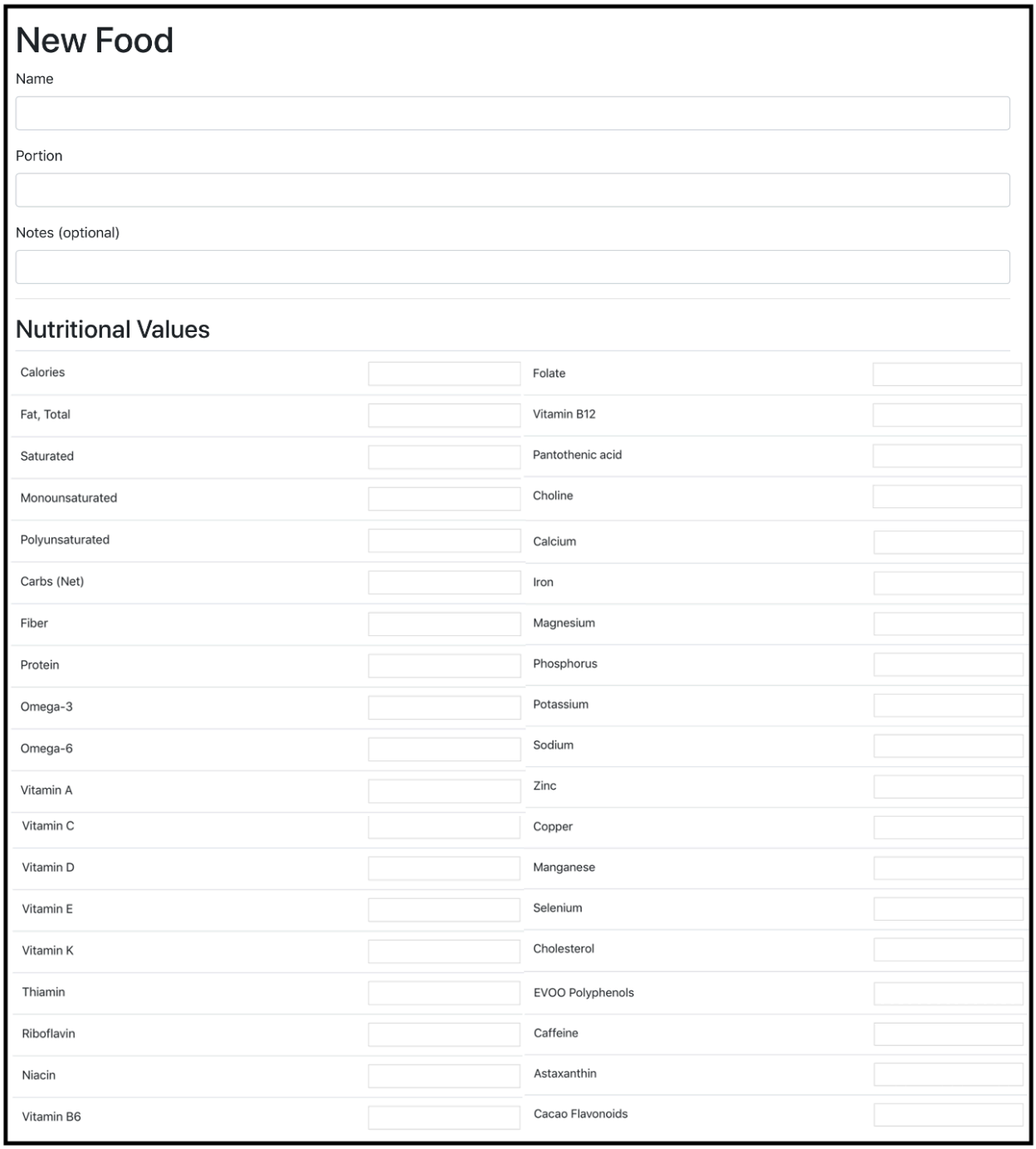


Figure 6.3 Food Library

When users enter a New Food into their Food Libraries, they are prompted to enter in data for each of the Goals.

6.3.2d Meal Library

To further ease tracking, individual Food Library items can be combined into meals or recipes in the "Meal Library." To add a new meal, researcher or participant users need only to click "New Meal" and then input the recipe of interest. While tracking, meals operate just like individual foods. Thus, tracking becomes easier because participants do not have to repeatedly input the components of meals they consume on a regular basis, and they can also consume fractions of larger recipes without having to calculate how much of each ingredient they consumed (Figure 6.4).

In review, the net effects of the fundamental personalizable aspects of MUNCH are to:

(i) improve the breath of parameters that can be measured through Goals and Meta Goals

(ii) improve accuracy of data being sourced through the Food Library

(iii) improve the accuracy of data input by participants through the customizable units

(iv) improve participant compliance through the Libraries and customizable units

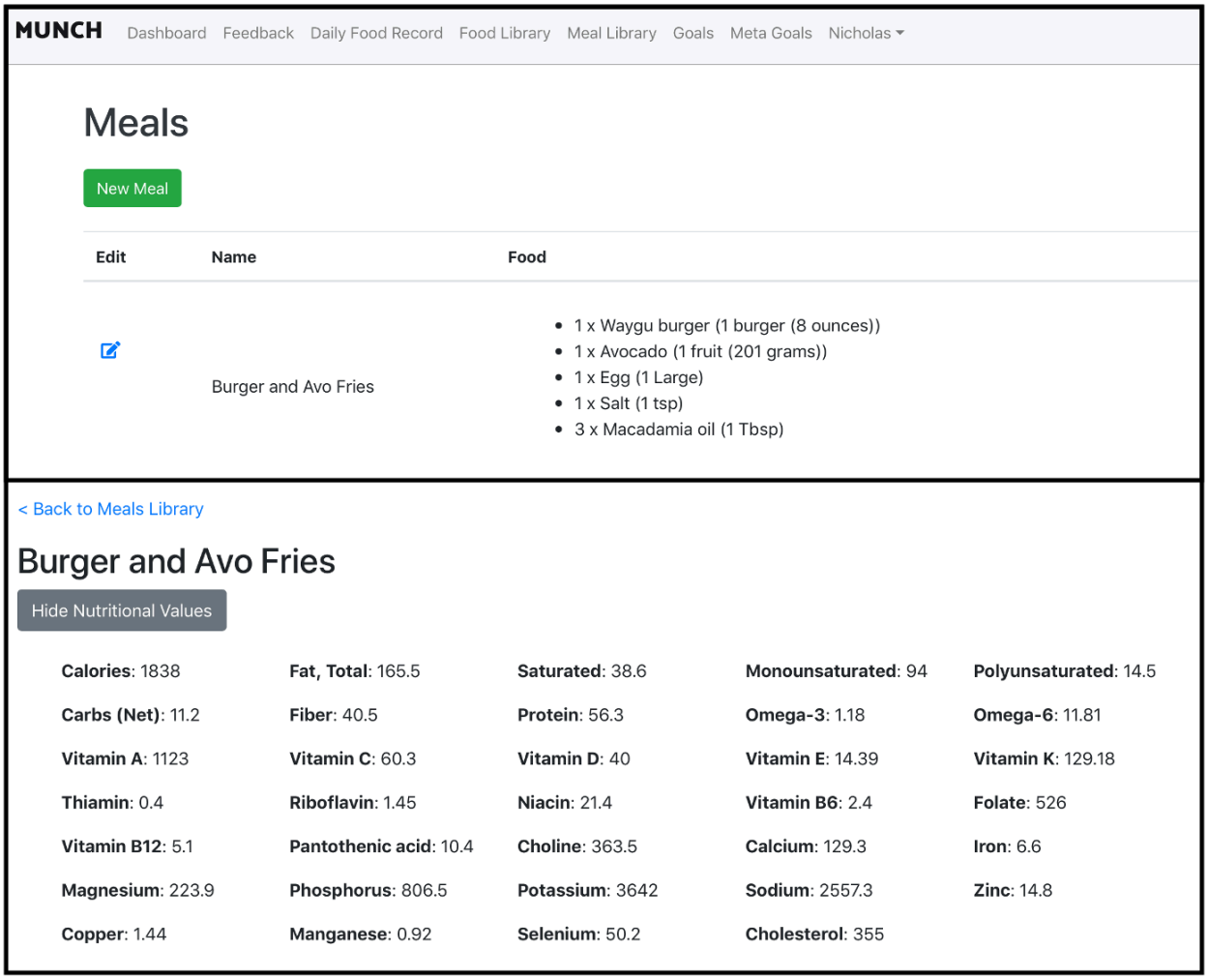


Figure 6.4 Meal Library

Users can create meals made up of individual foods from within the Food Libraries. The top panel shows the meal, "Burger and Avo Fries," in the Meal Library. The bottom panel shows shown the nutrition information for this meal. Meals act like foods when participants are tracking, thus expediting the data recording process.

6.3.2e Tracking and Daily Food Record

To record foods or meals in the "Daily Food Record," participants "Add Food or Meal" and then select a food or meal to add from a searchable dropdown list of their Food Library. Foods and meals are also timestamped, with the default time being the time of data entry (Figure 6.5).

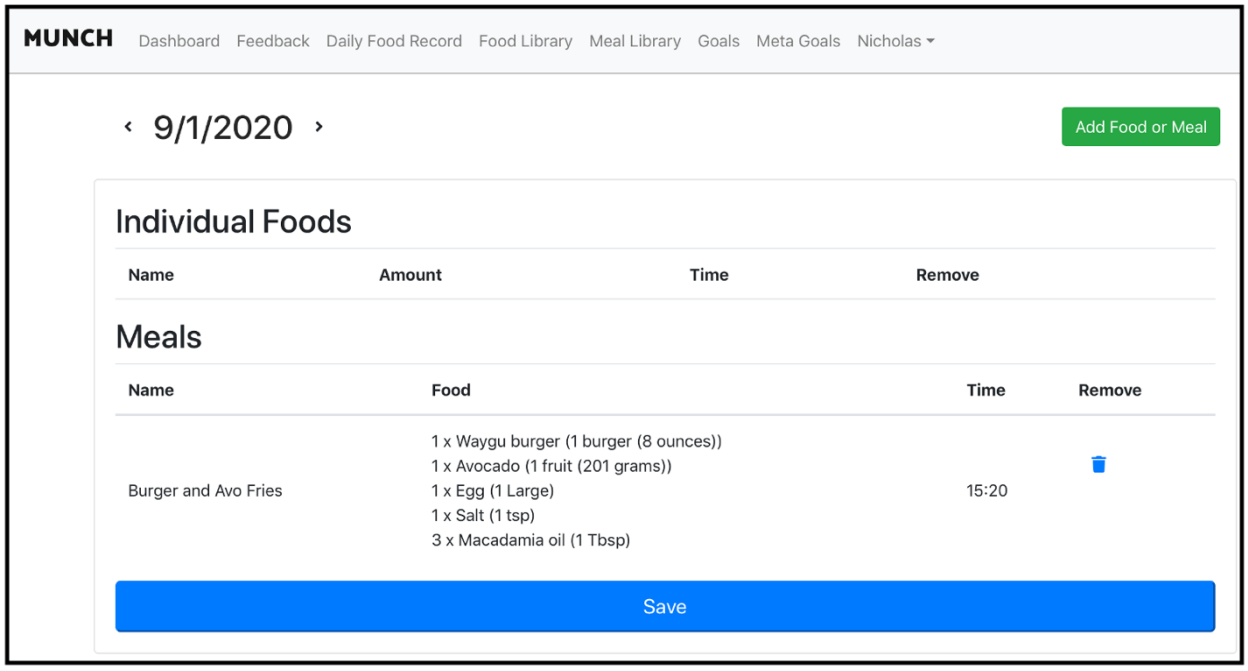


Figure 6.5 Tracking

To record intake, participants simply click "Add Food or Meal" (green button) and select foods from their libraries. In the example, the participant ate the "Burger and Avo Fries" meal.

6.3.2f Dashboard

Data for a select time period – days, weeks, months, or years – are displayed on the dashboard (see upcoming example).

6.4 Result: Example

To illustrate the functionalities and advantages of the MUNCH platform over existing nutrition tracking applications, this section provides a compare and contrast example between MUNCH and the top-rated low-carb diet tracking application, CarbManager.

The example meal consists of two large eggs from pastured hens, a pre-portioned frozen fillet of wild Alaskan sockeye salmon, one-third cup of Alta Cresta premium extra virgin olive oil, half a teaspoon of LoSalt, one-third package of arugula lettuce from Star Market, one medium (200 gram) avocado, and a bar of Evolved Midnight Coconut cacao-based 100% dark chocolate (Figure 6.6i).

Moving sequentially through the aforementioned foods:

Eggs: There are no options available on CarbManager for differentiating among conventionally raised, free-range, organic, omega-3-rich, or pastured eggs. This undermines data accuracy because hen rearing methods significantly influence eggs' nutritional quality, including vitamin E content and omega-3/6 ratio [144].

Salmon: The only option available on CarbManager for salmon is farmed Atlantic salmon. Similar to the situation with the eggs, farmed Atlantic salmon has meaningfully different fat content, both total and proportions of different fatty acids, and meaningfully different micronutrient content than wild Alaskan sockeye salmon. As a case in point, sockeye salmon contains as much as four-fold the astaxanthin as farmed Atlantic salmon [145]. Astaxanthin is a well-studied carotenoid pigment

that gives salmon their color and is thought to possess benefits for brain health [146, 147]. It is, therefore, relevant to track and track accurately. (Additionally, on CarbManager, the user needs to input a mass for the salmon fillet, whereas, on MUNCH, one fillet can be pre-set as a unit itself based on the particular packaged frozen sockeye salmon that the example user purchases.)

Olive oil: Olive oils vary widely in terms of quality, with a majority of extra virgin oils on the American and English markets being mislabelled as extra virgin, and sometimes mislabelled as olive oil [148]. Further, oils differ with respect to their polyphenol contents, which can be tracked only on MUNCH. In this example, the Alta Cresta brand oil that the user consumes has 483 mg/kg polyphenols, information sourced directly from the supplier's website [149].

Arugula lettuce: When recording arugula lettuce on CarbManager, the example user enters "lettuce" ("arugula" is not an option) and guesses at a portion of one cup. This leads to inaccuracies in nutrient density and intake volume. Arugula is a cruciferous vegetable, similar to broccoli or Brussels sprouts, with a different nutrition profile than true lettuces like romaine. As a cruciferous vegetable, arugula is rich in is rich in glucosinolates, sulphur-containing compounds that have been studied for their anti-carcinogenic properties and other impacts on metabolism [150, 151]. Arugula also contains five-fold as much calcium but one-third as much vitamin A as romaine. Furthermore, the one cup unit is problematic as this serving size is not intuitive when applied to lettuce and cannot be easily measured using a measuring cup. By contrast, on MUNCH, the participant simply enters a proportion of the package of arugula that s/he purchases. The result is more accurate data, both because of the input options and input quantity.

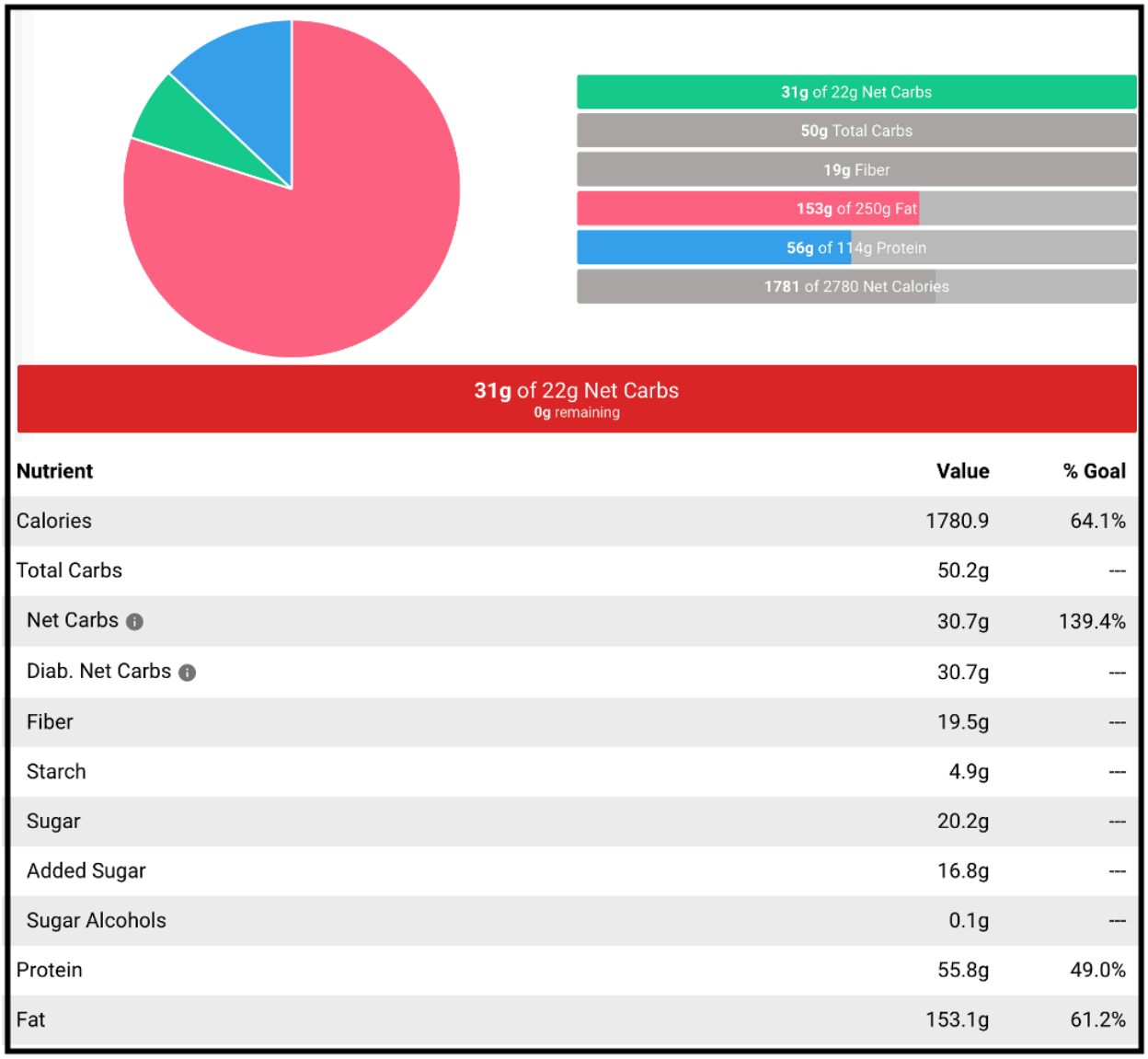
Avocado: CarbManager has the user to simply enter “1 each” as a unit, without differentiating among avocados of different sizes. On MUNCH, nutrition information for avocado is set to correspond to the Hass avocado variety and size of avocado (medium, approximately 200 grams) that the user purchases. With respect to avocados, the size factor alone can account for a difference of up to about 200 Calories, not to mention the proportional impact on macronutrient and micronutrient intakes.

Chocolate: CarbManager does not provide an option to enter 100% dark chocolate, let alone cacao-based chocolate or the Evolved brand bar that the example user purchases. Instead, the user must select the darkest chocolate option, which is 70-85% dark chocolate, as well as a unit (2.6-ounce “Large Bar”) that most closely approximates the 2.3-ounce Evolved bar. The net carbohydrate difference between the two inputs amounts to 19.1 grams of net carbohydrates. For someone on a ketogenic diet, this is roughly one entire day’s allocation being fulfilled by error.

The resulting data outputs for the same example meal on the two platforms are shown in Figure 6.6ii-iii, and the two platforms are directly compared in Table 6.1.

Figure 6.6i Comparison between CarbManager and MUNCH – Example

The example meal consists of two large eggs from pastured hens, a pre-portioned frozen fillet of wild Alaskan sockeye salmon, one-third cup of Alta Cresta premium extra virgin olive oil, half a teaspoon of LoSalt, one-third package of arugula lettuce from Star Market, one medium (200 gram) avocado, and a bar of Evolved Midnight Coconut cacao-based 100% dark chocolate.



Nutrient	Value	% Goal
Calories	1780.9	64.1%
Total Carbs	50.2g	---
Net Carbs	30.7g	139.4%
Diab. Net Carbs	30.7g	---
Fiber	19.5g	---
Starch	4.9g	---
Sugar	20.2g	---
Added Sugar	16.8g	---
Sugar Alcohols	0.1g	---
Protein	55.8g	49.0%
Fat	153.1g	61.2%

Figure 6.6ii Comparison between CarbManager and MUNCH – CarbManager

CarbManager output for the example meal is necessarily inaccurate because it does not provide options for certain foods that the example user consumed. For example, the calculated net carbohydrate intake is three-fold greater than what was consumed and, as a result, the example user is misinformed that s/he has exceed her/his daily net carbohydrate allowance. CarbManager also provides a limited breath of data and does not provide options for tracking important nutrient variables, such as polyphenols or omega-6/3 ratio.

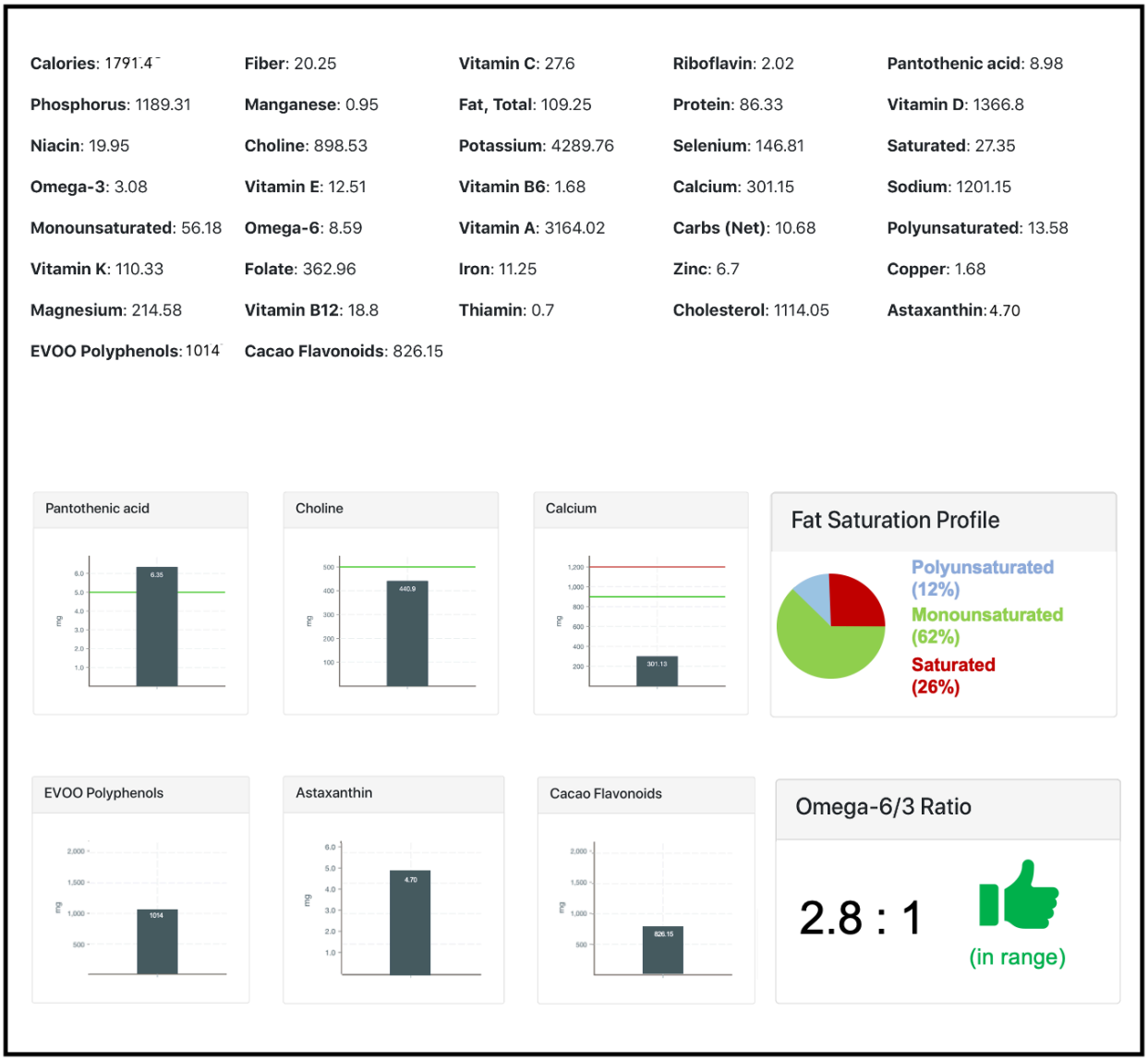


Figure 6.6iii Comparison between CarbManager and MUNCH – MUNCH

MUNCH output for the example meal is more accurate and provides a wide array of data. As examples, MUNCH calculates a net carbohydrate intake of 10.68 grams, as compared to CarbManager's 31 grams, and provides data on various polyphenols and omega-6/3 ratio.

Category	CarbManager #1 Nutrient Tracking App of 2019	MUNCH Version 1
Breath	★★★ Macronutrients and a limited number of micronutrients	★★★★★ Infinite parameters
Accuracy	★★★ 2-20% error in Calories (n = 10 tests)	★★★★★ Precise, based on brands/varieties individual consumes
Flexibility	Parameters are pre-set	★★★★★ Define new parameters of interest
Usability	★★ Portion sizes are potentially confusing	★★★★★ Better usability because portion sizes are defined by the individual
Time	★ 5-10 minutes	★★★★★ <5 minutes (new meal) <1 minute (saved meal)
Personalized Advice	N/A	Innovation, enabled by flexible platform

Table 6.1 Comparison between CarbManager and MUNCH

Unlike CarbManager, and other existing nutrition tracking applications, MUNCH allows for the tracking of an infinite number of parameters (breath), provides data based on specific foods that participants consume (accuracy), allows researchers to define parameters that are of interest to them (flexibility), employs portion sizes that are intuitive for participants (usability), and is quicker to use when recording data (time). The flexibility of MUNCH also enables participants or patients to receive personalized advice and real-time feedback to keep them within their targets and improve their nutrition habits (see next section).

6.5 Future Directions for MUNCH

6.5.1 Prescription digital therapeutic

MUNCH can also be used as a clinical tool, or prescription digital therapeutic (PDT). PDTs are software-based disease management strategies that have attracted interest as non-pharmacological methods of treating chronic diseases.

The customizability of MUNCH makes it a perfect basis for a PDT. Clinicians can customize Goals and Food Libraries for, and with the input of, patients based on their specific conditions. Perhaps even more importantly, because the nutrient Goals and targets are specified for individual patients, and the Food Library is built from foods patients actually consume, MUNCH can provide personalized and real-time feedback to help patients stay within target ranges for nutrients, as defined by their clinicians.

For example, if a clinician aims to optimize a patient's omega-6/3 ratio to help reduce inflammation and oxidative stress that may be exacerbating her/his chronic disease, MUNCH would not only tell the patient when s/he moves out of range but would also provide recommendations on how to return to the ideal range. In this instance, MUNCH may observe that the patient was consuming a large amount of walnuts, which contain over 50% omega-6 fat content, and also that the same patient enjoys macadamia nuts, which are only 2% omega-6 content. Therefore, MUNCH could recommend that the patient substitute the former nut for the latter nut to optimize omega-6 intake.

As a second example, perhaps a participant is a known carrier of the methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphism, *C667T*, which has a high minor allele frequency of 32%, decreases enzyme activity by as much as 70% in homozygotes [152], and confers an increased risk for vascular disorders and for neurological and neurodegenerative diseases [153-155]. This polymorphism acts by impairing methylation cycle function and increasing plasma homocysteine levels, a defect that can be treated by simply providing higher levels of bioavailable B-vitamins that support the methylation cycle, such as riboflavin [156], 5-methyltetrahydrofolate [157], and methylcobalamin [158]. Thus, a clinician treating a patient carrying this common *MTHFR* allele may choose to increase the patient's "minimum" of these B-vitamins in order to improve disease risk. MUNCH would provide real-time feedback and make recommendations to keep the patient above her/his personalized, clinician-set threshold.

6.5.2 Additional features

At the current time, MUNCH is not available as a scalable tool, but only as an online version 1 prototype to demonstrate the concept. Future generations of MUNCH will incorporate additional features that will improve usability for researchers, research participants, clinicians, and patients.

6.5.2a Importing and sharing data

The evident barrier to using MUNCH is the building of the Food Library, as researchers or clinicians need to enter data for each variable of interest. This process can be expedited in future by enabling the import of data directly from verified sources. This need not undermine the accuracy of MUNCH data because it would be an optional feature that the user employs when there are appropriate options available on such databases. For example, while CarbManager may not be able

to provide data for sockeye salmon, the SR legacy database on the United States Department of Agriculture's Food Central website does include such an entry. Furthermore, those data could subsequently be updated within MUNCH based on nutrition labels or information on nutrients, such as astaxanthin, available in the published literature [145].

In addition to importing and editing data from publicly available databases, future generations of MUNCH will allow users to exchange entries and data. Over the short-term, this will enable researchers and clinicians to more easily build accurate Food Libraries for multiple participants and patients. Over the long-term, such a sharing model could form the basis of an independent and comprehensive database of foods and their nutrients. The importing and sharing data features will make MUNCH a more user-friendly tool from the point of view of researchers and clinicians.

6.5.2b Integration with biological data

Personalized medicine is the future. More and more, genetic, metabolic, and microbiome data are available for research and clinical purposes. MUNCH complements the personalized medicine trajectory and could eventually integrate such data in order to investigate the relationships among the intake of particular nutrients and changes in biological parameters. MUNCH could be used to probe how particular genetic mutations alter need for particular micronutrients, or how particular dietary changes alter the microbiome.

Not only are the possibilities for research boundless, but a union between future versions of MUNCH and biological data could allow clinicians to better customize nutrient targets for their patients. For example, imagine it were discovered that a particular bacterial species

underrepresented in a patient's gut is supported by vitamin K intake and is associated with improvements in her/his symptoms. In this instance, a clinician, instead of recommending a supplement, could adjust the patient's minimum target for vitamin K such that MUNCH advises her/him on how to get more vitamin K in the diet. MUNCH would then further continually update its feedback based on observations of how real-world dietary changes in the individual alter her/his microbiome.

6.6 Summary

Metabolic diseases (including, but not exclusively, neurodegenerative and neurological diseases) necessitate metabolic treatments. Nutrition is one form of metabolic treatment; however, new nutritional treatments cannot be developed without strong nutrition research. The purpose of MUNCH is to enable a new form of nutrition research to advance the state of nutrition science. MUNCH can also serve as a PDT to help clinicians directly treat their patients with nutrition. Although it is currently only a version 1 model, the unique flexible approach of the MUNCH platform demonstrates that it fulfils a niche in the field of nutrition research and that it has potential to develop into a beneficial tool for nutrition science and medicine.

Chapter 7. *Seven review articles and theory papers on ketogenics and neurodegenerative and neurological diseases (peer-reviewed and published)*

Declaration

The following chapter is composed of six published peer-reviewed articles. I am the first author on five of these publications, and, on these, I certify that the texts represent entirely my original ideas and are written in my own words. General editorial thoughts by my co-authors were considered and incorporated where appropriate.

I include this declaration to reinforce that I understand the University of Oxford's policy on plagiarism and certify that this thesis represents my own work, expect where appropriately indicated.

7.1 **Norwitz NG**, Hu MT, Clarke K. The mechanisms by which the ketone body D-β-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease. *Frontiers in Nutrition*. 2019

7.1.1 Statement of relevancy

The following manuscript was intended to serve as molecular justification for the hypothesis that βHB may act as a disease-modifying agent for Parkinson's disease.

7.1.2 Abstract

Parkinson's disease, a progressive neurodegenerative disorder characterized by motor and non-motor symptoms, is associated with the death of dopaminergic neurons in the brain's substantia nigra. Although dopamine replacement therapy temporarily helps patients manage their motor symptoms, this current standard of care fails to address the underlying network of pathologies that contribute to the persistent death of dopaminergic neurons. Thus, new treatment approaches are needed that address the underlying pathologies and, thereby, slow or halt the progression of the actual disease. D-β-hydroxybutyrate – a ketone body produced by the liver to support brain function during periods of starvation – may provide an option. Lifestyle interventions that induce endogenous D-β-hydroxybutyrate production, such as caloric restriction and ketogenic diets, are known to increase healthspan and lifespan in animal models and are used to treat neurological disorders. The efficacy of these ketosis-inducing interventions, along with the recent development of commercially available D-β-hydroxybutyrate-based nutritional supplements, should inspire interest in the possibility that D-β-hydroxybutyrate itself exerts neuroprotective effects.

This review provides a molecular model to justify the further exploration of such a possibility. Herein, we explore the cellular mechanisms by which the ketone body, D-β-hydroxybutyrate, acting both as a metabolite and as a signaling molecule, could help to prevent the development, or slow the progression of, Parkinson's disease. Specifically, the metabolism of D-β-hydroxybutyrate may help neurons replenish their depleted ATP stores and protect neurons against oxidative damage. As a G-protein-coupled receptor ligand and histone deacetylase inhibitor, D-β-hydroxybutyrate may further protect neurons against energy deficit and oxidative stress, while also decreasing damaging neuroinflammation and death by apoptosis. Restricted to the available evidence, our model relies largely upon the interpretation of data from the separate literatures on the cellular effects of D-β-hydroxybutyrate and on the pathogenesis of Parkinson's disease. Future studies are needed to reveal whether D-β-hydroxybutyrate actually has the potential to serve as an adjunctive nutritional therapy for Parkinson's disease.

7.1.3 Introduction

Parkinson's disease (PD) is the world's second most common and fastest growing neurodegenerative disorder. At present, PD affects 2–3% of individuals over the age of 65, a figure that is expected to double by the year 2040 [8, 9]. Hence, if it were an infectious disease, PD would quite rightly be called a pandemic. Symptomatically, PD manifests in several classical motor symptoms, including tremors and bradykinesia, as well as in a wide variety of non-motor symptoms, such as disordered sleep and cognitive dysfunction. As there is no cure for PD, symptoms inevitably progress and inflict devastating consequences on individuals and on their families.

In common with Alzheimer's disease and other neurodegenerative diseases, PD is biologically characterized by protein misfolding and the rampant death of neurons. Specifically, PD is characterized by the aggregation of α-synuclein protein and the death of dopaminergic neurons in the midbrain substantia nigra (SN), although PD affects other neurotransmitter systems as well. The pathological mechanisms underlying these biological hallmarks are highly complex and include both metabolic and signaling dysfunctions.

As both a fuel substrate and signaling molecule, the ketone body D-β-hydroxybutyrate (βHB) may be well-suited to slow, halt, or even reverse the progression of PD. Indeed, Tieu et al. have shown that that βHB can successfully protect against the death of dopaminergic neurons in the SN and that βHB can alleviate the symptoms of PD in mice [81]. At present, many publications are available describing either the ways in which βHB alters metabolism/cell signaling or how βHB affects specific pathological mechanisms that are associated with PD. This review attempts to

synthesize these separate bodies of evidence in order to suggest a model for how βHB, acting as both a metabolite and as a signaling molecule, might address many aspects of the pathological network underlying PD; and, therefore, that exogenous βHB supplementation may represent an improvement upon the current standard of care. In this review, we subcategorize βHB's effects on metabolism and cell signaling, respectively, into βHB's effects on (i) ATP production and (ii) antioxidant defences, and into βHB's actions as (iii) an activator of the G-protein-coupled, hydroxycarboxylic acid receptor 2 (HCAR2) and as (iv) an inhibitor of histone deacetylases (HDACs). Throughout the review we attempt to identify how βHB, acting through these four strategies, can protect against the four most prominent pathological mechanisms in PD, namely abnormal cellular energy metabolism, oxidative stress, inflammation, and apoptosis (Figure 7.1).

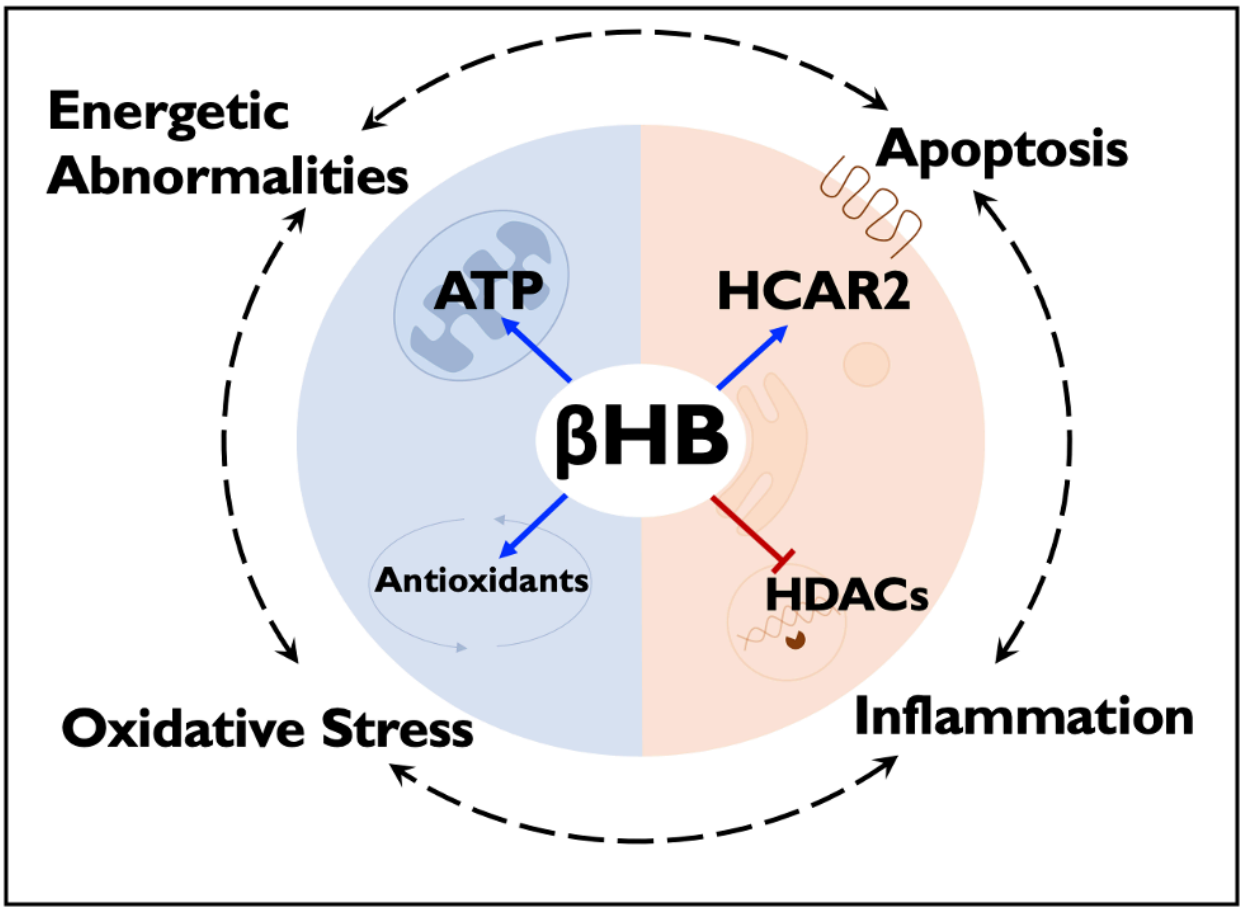


Figure 7.1 βHB protects against Parkinson's disease pathologies

βHB acts as a metabolite (blue), to increase mitochondrial ATP production and bolster antioxidant defences, and as a signaling molecule (orange), to activate the G-protein-coupled, hydroxycarboxylic acid receptor 2 (HCAR2) and inhibit class I and II histone deacetylases (HDACs), thereby targeting the four fundamental pathologies underlying PD, as well as most other neurodegenerative and neurological diseases.

7.1.4 Abnormal energy metabolism

Due to their large size, extensive arborization and calcium-pacemaking activity, SN dopaminergic neurons are particularly metabolically active cells and are, therefore, especially susceptible to energy deprivation [8, 88]. Indeed, ATP levels in the region of the brain that includes the SN are reduced by as much as 44% in the brains of patients with PD as compared to age-matched controls [36]. Evidence from mouse models suggests that this relationship is not simply correlative. MPTP, the complex I inhibitor most commonly used to produce animal models of PD, depletes cerebral ATP *in vivo* [159]. Furthermore, pharmacologically blocking ATP consumption or increasing ATP production in PD mice is sufficient to prevent α-synuclein aggregation and the death of dopaminergic neurons in the SN, and is sufficient to protect against the motor symptoms of PD [89].

There are at least two mechanisms by which βHB may increase ATP levels in dopaminergic neurons. First, the work that launched contemporary scientific interest into the field of exogenous ketones, conducted by Sato et al. on perfused rat hearts, suggests that βHB metabolism increases mitochondrial ATP production by exerting opposite redox effects on the respiratory chain electron carriers, NAD and coenzyme Q (Q). By reducing (decreasing) the mitochondrial NAD^+/NADH ratio, while simultaneously oxidizing (increasing) the Q/QH_2 ratio, βHB increases the difference between the redox potentials of these two electron carrier couples [80]. This increase in redox span is biochemically analogous to increasing the height span from which a bowling ball is dropped to the ground. In both cases, more energy is available to do work. Therefore, when electrons are passed down from NADH to Q, more protons can be pumped into the intermembrane space to drive the generation of more ATP by chemiosmosis. In this way, βHB metabolism can increase

the redox span within the electron transport chain to increase the generation of ATP by oxidative phosphorylation (Figure 7.2).

Rodent data suggest that βHB metabolism also permits dopaminergic neurons to circumvent the blockade of complex I, a phenomenon that contributes to mitochondrial dysfunction in human PD [72, 160, 161], by feeding electrons into the respiratory chain at complex II (Figure 7.2). This mechanism makes biochemical sense because the rate limiting step of βHB catabolism generates succinate, the oxidative fuel for complex II. Specifically, it has been shown that administration of βHB to MPTP-treated PD mice protects dopaminergic SN neurons from cell death and that this effect is blocked by the specific inhibition of complex II [81]. In addition, βHB is able to increase ATP levels in brain mitochondria in the presence of MPTP-mediated complex I inhibition, but not when flux through complex I and complex II are both inhibited [81].

And, although flux through complex II can be linked to a decreased Q/QH_2 ratio, the redox span and complex II flux models are not contradictory because an increase in flux through a pathway does not equate to an increase in the metabolites in that pathway. In fact, βHB only increases succinate levels when flux through complex II is blocked [81], a finding consistent with the notion that βHB can increase Q/QH_2 turnover without decreasing the ratio itself. Therefore, these two mechanisms, whereby βHB increases ATP production in PD, may be less contradictory than complimentary.

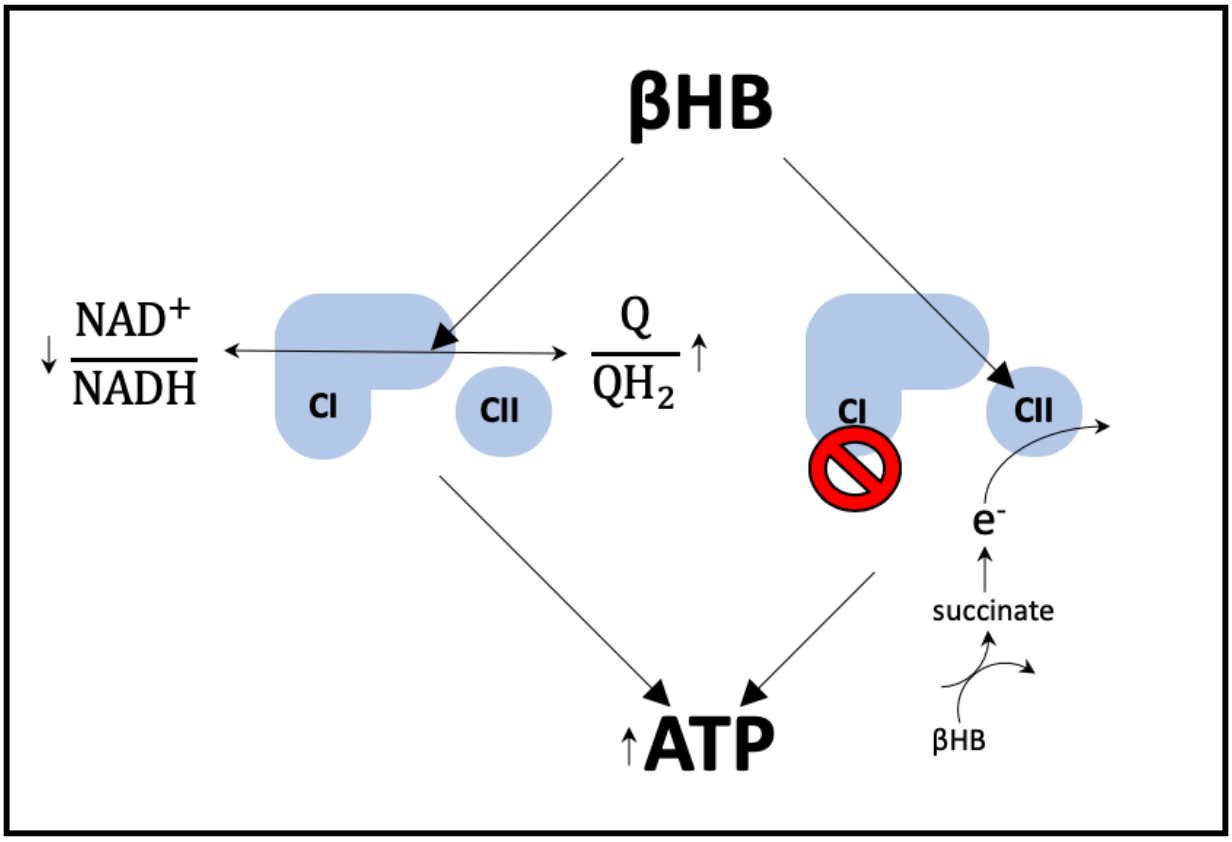


Figure 7.2 βHB improves energetics

βHB decreases the NAD^+/NADH ratio and increases the Q/QH_2 ratio, resulting in an increase in the redox span between the two couples. More energy is liberated by the transfer of electrons from NADH to Q and, thereby, ATP production is increased. βHB also acts to circumvent the pathological blockade of complex I (CI) observed in PD by increasing flux through complex II (CII) via the production of succinate.

7.1.5 Oxidative stress and antioxidants

By altering the ratios of redox couples within mitochondria and the cytoplasm, βHB metabolism may both diminish the production of reactive oxygen species (ROS) and bolster antioxidant defences. Most mitochondrial ROS are generated via the process of reverse electron transport, in which electrons are passed from QH_2 to oxygen at complex I to generate superoxide. Therefore, by oxidizing the Q/QH_2 couple [80], βHB not only increases the production of ATP, but also decreases the production of ROS (Figure 7.3).

In addition to increasing the Q/QH_2 ratio and decreasing the NAD^+/NADH ratio [80], βHB also decreases the $NADP^+$ /NADPH ratio through a variety of mechanisms. First, NADH and NADPH are tightly linked, especially in neurons, by the direct transfer of hydride ions from NADH to $NADP^+$ via nicotinamide nucleotide transhydrogenase [106, 107]. Second, by decreasing glycolytic flux, βHB forces glucose-6-phosphate down the pentose phosphate pathway, leading to the production of two equivalents of NADPH [80, 106-108]. Third, by increasing ~15-fold the concentration of mitochondrial acetyl-CoA [80], βHB increases the concentration of mitochondrial citrate and the export of this citrate into the cytoplasm by the citrate-pyruvate and citrate-isocitrate cycles, each of which includes an $NADP^+$ to NADPH reduction step catalyzed by malic enzyme and isocitrate dehydrogenase, respectively [106, 109]. The products of these two carrier systems, oxaloacetate and α-ketoglutarate, are returned to the Krebs cycle to complete the circuit. Therefore, by increasing mitochondrial citrate concentrations, βHB forces the citrate-pyruvate and citrate-isocitrate wheels to spin faster to produce more NADPH (Figure 7.3). As all known intracellular antioxidants, either directly or indirectly, depend upon NADPH as the electron donor, βHB-

mediated reduction of $NADP^+$ into NADPH translates into an increase in the reduced levels of glutathione, thioredoxin, vitamins C and E, and other essential antioxidants [109].

By increasing NADPH levels, βHB may exert another beneficial effect in the context of PD: βHB may increase the synthesis of dopamine and other neurotransmitters. As the ultimate reducing agent, NADPH supports the reduction of dihydrobiopterin into tetrahydrobiopterin, a coenzyme critical in the synthesis of dopamine, noradrenalin, serotonin, and melatonin. Although the possible effect of βHB on these neurotransmitter systems is speculative and requires further investigation, it is worth mentioning if for no other reason than this mechanism by which βHB might increase neurotransmitter levels is distinct from the current standard of care for PD, levodopa therapy, which only provides the direct precursor to dopamine. Therefore, βHB may be able to increase the synthesis, not only of dopamine [81], but also of other neurotransmitters that are underproduced in PD and which contribute to the non-motor symptoms of PD not addressed by levodopa therapy [109].

βHB not only increases the Q/QH_2 ratio to decrease the generation of ROS, and decreases the $NADP^+$/NADPH ratio to increase antioxidant defences and support neurotransmitter synthesis, but also has the unique ability to regulate all four of the "great" controlling nucleotide coenzyme couples: NAD^+ /NADH, $NADP^+$ /NADPH, acetyl-CoA/CoA, and ATP/ADP. These coenzymes diffuse throughout the cell, collectively regulating at least 1,500 known enzymatic reactions through their shared potential energies. Therefore, by regulating these four couples, βHB metabolism shapes the entire metabolome in ways that we are only beginning to appreciate and may affect not only energy crisis and oxidative stress, but also inflammation and apoptosis [109].

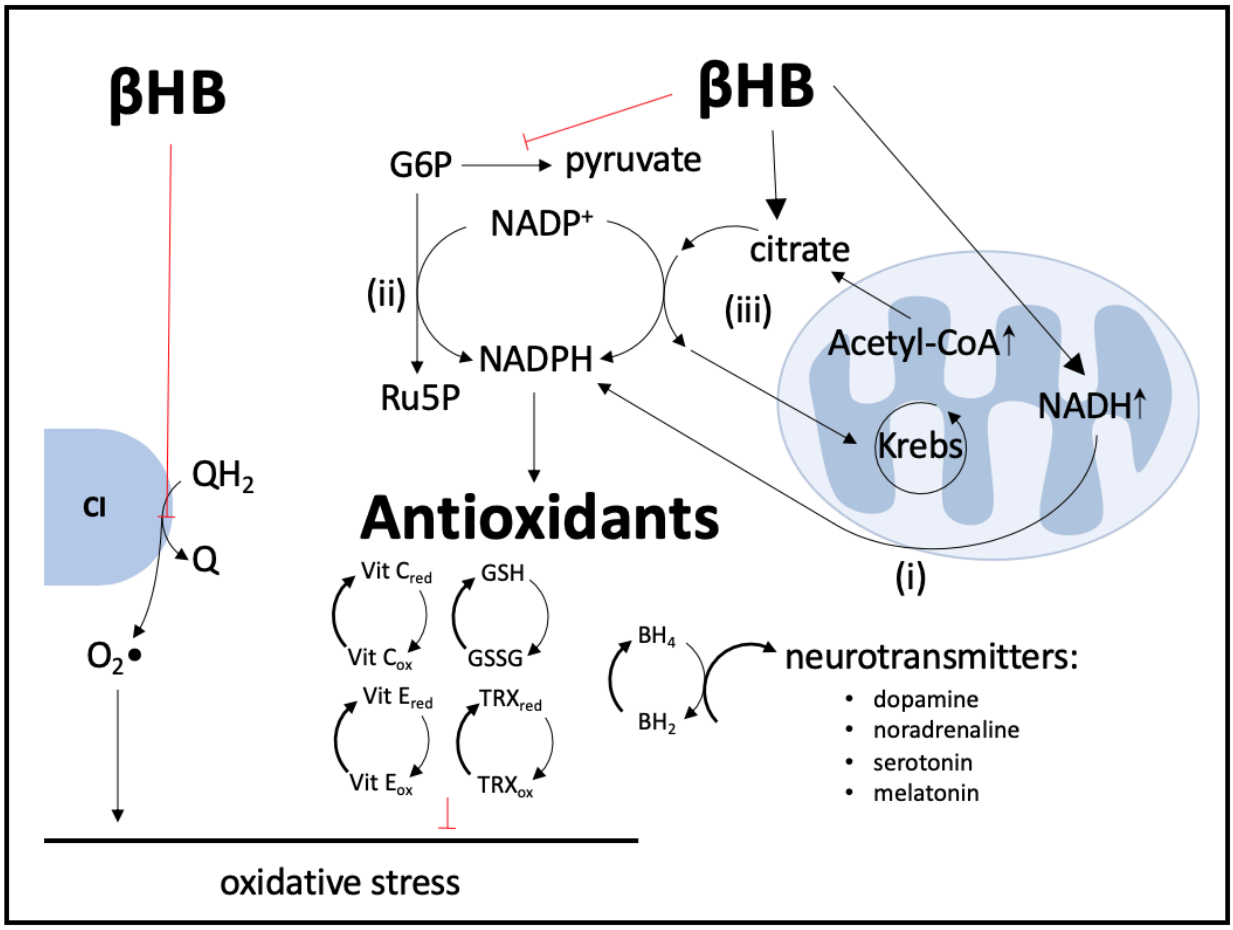


Figure 7.3 βHB decreases reactive oxygen species (ROS) production, increases antioxidant defences and increases neurotransmitter synthesis

βHB oxidizes the Q/QH_2 couple to decrease the transfer of electrons from QH_2 to oxygen by reverse electron transport at complex I (CI) and, thus, decrease the production of superoxide (O2•) radicals. βHB generates NADPH by (i) increasing the transfer of hydride ions from NADH to $NADP^+$, (ii) inhibiting glycolysis, thus increasing pentose phosphate pathway flux, and (iii) increasing citrate-pyruvate and citrate-isocitrate cycle flux. NADPH, in turn, supports antioxidant defences, including the glutathione (GSH-GSSG), thioredoxin (TRX) and vitamins C and E. NADPH reduces dihydrobiopterin (BH_2) into tetrahydrobiopterin (BH_4) and, thereby, increases the synthesis of the neurotransmitters dopamine, noradrenaline, serotonin and melatonin.

7.1.6 G-protein coupled receptor (HCAR2) activation

In addition to its role as a fuel substrate, βHB is a ligand for hydroxycarboxylic acid receptor 2 (HCAR2), a G-protein coupled receptor that is upregulated in the SN of PD patients [108, 162, 163]. Although HCAR2 is upregulated in the PD brain, it is predicted to be underactive because PD patients also exhibit lower levels niacin, another HCAR2 ligand [163]. Therefore, by substituting for niacin and activating upregulated HCAR2, βHB may be able to target a set of sensitized pathways in the PD brain, pathways that include the critical proteins SIRT1 and NFκB (Figure 7.4).

SIRT1 is a deacetylase whose activity is strongly associated with the generic health and longevity benefits of caloric restriction and that is underactive in the PD brain [164, 165]. One way in which SIRT1 is thought to mediate its neurological benefits is by upregulating autophagy, a catabolic process active in all cells that facilities the degradation and recycling of damaged cellular components. SIRT1 activates a handful of autophagy proteins, including ULK1 and several ATGs, to increase the activity of this essential cellular recycling process and promote the clearance of defective and damaged mitochondria [166, 167]. In a complementary fashion, SIRT1 activates the master regulator of mitochondrial biogenesis, PGC1-α, which is also underexpressed in, and has been strongly implicated in the pathogenesis of PD [166, 168-170]. Not only can SIRT1 increase autophagy and mitochondrial biogenesis, but it also induces FOXO3A-dependent expression of the antioxidant genes catalase, mnSOD, and Mt2 [166]. Therefore, the stimulation of SIRT1 by βHB could protect cells against energy depletion and oxidative stress by improving the health of the mitochondrial pool and by bolstering antioxidant defences. In addition, rodent studies suggest that a ketogenic diet induces SIRT1-dependent deacetylation of the transcription factor p53 and,

thereby, increases the expression of anti-apoptotic proteins, decreases the expression of pro-apoptotic proteins, and ultimately protects neurons from apoptosis [122] (Figure 7.4).

By binding to HCAR2 on macrophages and microglia in the brain, βHB also inhibits NFκB-mediated neuroinflammation, a critical pathological feature in PD [171-173] (Figure 7.4). NFκB is a potent proinflammatory transcription factor that is elevated in the PD brain [174], has been proposed as a therapeutic target for PD [175], and induces the expression of TNF-α, IL-1β, and inducible nitric oxide synthase (iNOS) [176-178]. In addition to stimulating an innate immune response in the brain, the cytokines TNF-α and IL-1β, which have likewise been proposed as targets for potential PD treatments [179], can also directly promote apoptosis by binding to death receptors on neurons [180, 181]. The NO generated by iNOS, which is also pathologically important in PD [182], is likewise a proinflammatory molecule that exerts other pathological influences. NO can block glutamate reuptake, leading to excitotoxicity and apoptosis [177]. NO can also provoke oxidative stress by post-translationally modifying proteins important in mitochondrial quality control and can induce nitroxidative stress by combining with superoxide to form peroxynitrite and other cytotoxic molecules [177].

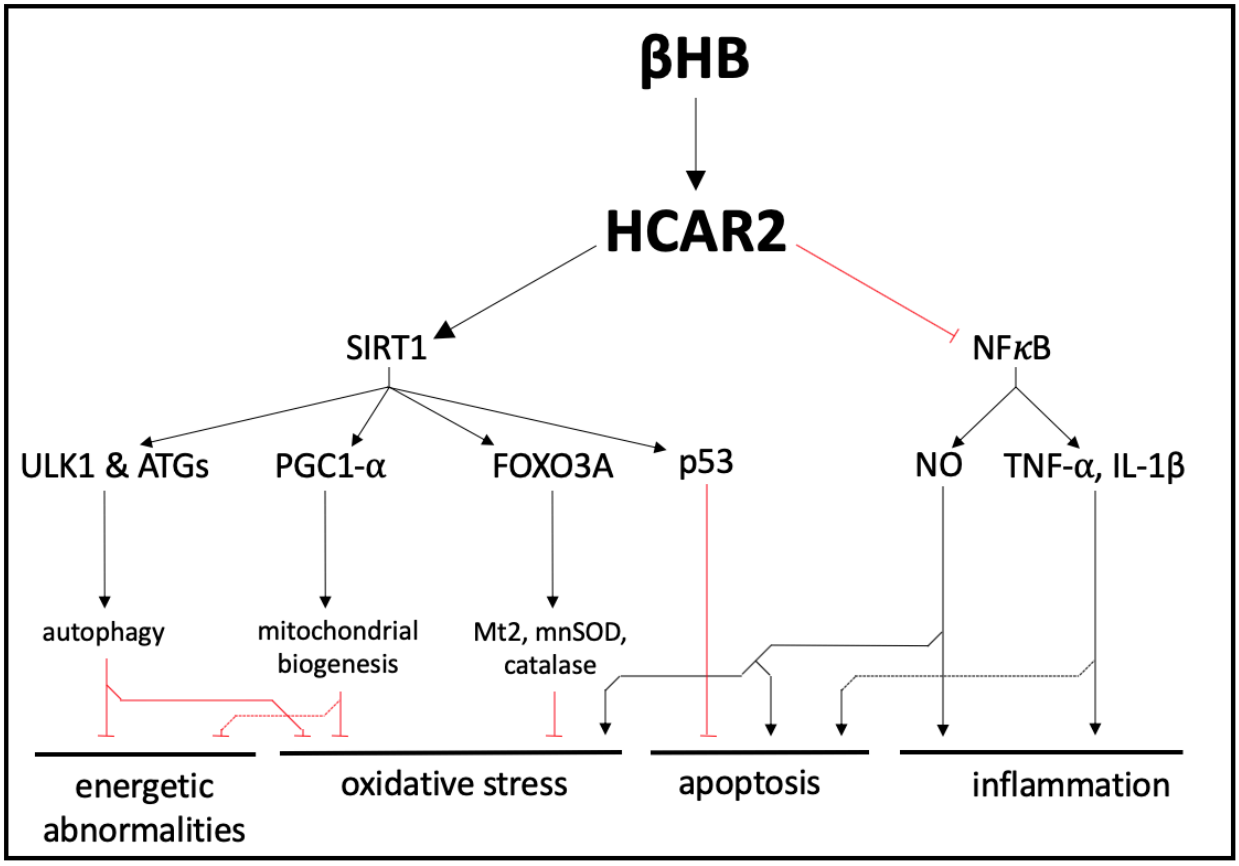


Figure 7.4 βHB exerts neuroprotective effects by activating hydroxycarboxylic acid receptor 2 (HCAR2)

Activation of HCAR2 promotes the downstream activation of SIRT1 and inhibition of NFκB to protect against the fundamental pathologies of PD.

7.1.7 Histone deacetylase inhibition

It has been shown *in vitro* that class I/II histone deacetylase (HDAC) inhibition decreases α-synuclein toxicity and protects dopaminergic neurons from cell death [183]. As a natural inhibitor of HDACs 1, 3, and 4 [162, 184], βHB may manage each of the pathologies underlying PD by regulating HDACs and altering gene expression.

For example, βHB-mediated HDAC inhibition in mice increases the expression of brain-derived neurotrophic factor (BDNF) [47], a molecule renowned for its putative ability to stimulate adult neurogenesis. In patients with PD, BDNF expression in the SN is significantly decreased [133], and, in rodent and primate models of PD, BDNF has been shown to protect dopaminergic SN neurons from cell death [185, 186]. In mouse primary cortical neurons, βHB-mediated HDAC inhibition increases BDNF expression, decreases the NAD^+/NADH ratio, and increases ATP [47, 85]. BDNF also prevents NFκB-mediated neuroinflammation and apoptosis in rodent models of central nervous system inflammatory diseases [187, 188]. Finally, BDNF increases the activity of multiple antioxidant enzymes and decreases oxidative damage in the basal ganglia of PD rats [189, 190]. Therefore, by increasing BDNF expression alone, βHB may protect against energy depletion, oxidative stress, inflammation, and apoptosis (Figure 7.5).

BDNF is one representative example of how βHB-mediated HDAC inhibition can exert a wide range of neuroprotective effects by altering transcription; another example being βHB-mediated upregulation of the antioxidant genes catalase, mnSOD, and Mt2 [162, 191] (Figure 7.5). In fact, the βHB target, HDAC4, is responsible for the characteristic pattern of gene expression that differentiates PD neurons from healthy neurons [184]. Furthermore, HDAC4 inhibition rescues

gene expression in dopaminergic neurons derived from patients with PD. This genetic rescue, in turn, decreases endoplasmic reticulum stress, which is a cellular response to protein aggregation marked by (i) oxidative stress, (ii) apoptosis [192, 193], and (iii) glucocerebrosidase degradation (a critical autophagy-related enzyme decreased in the PD brain) [194, 195] (Figure 7.5).

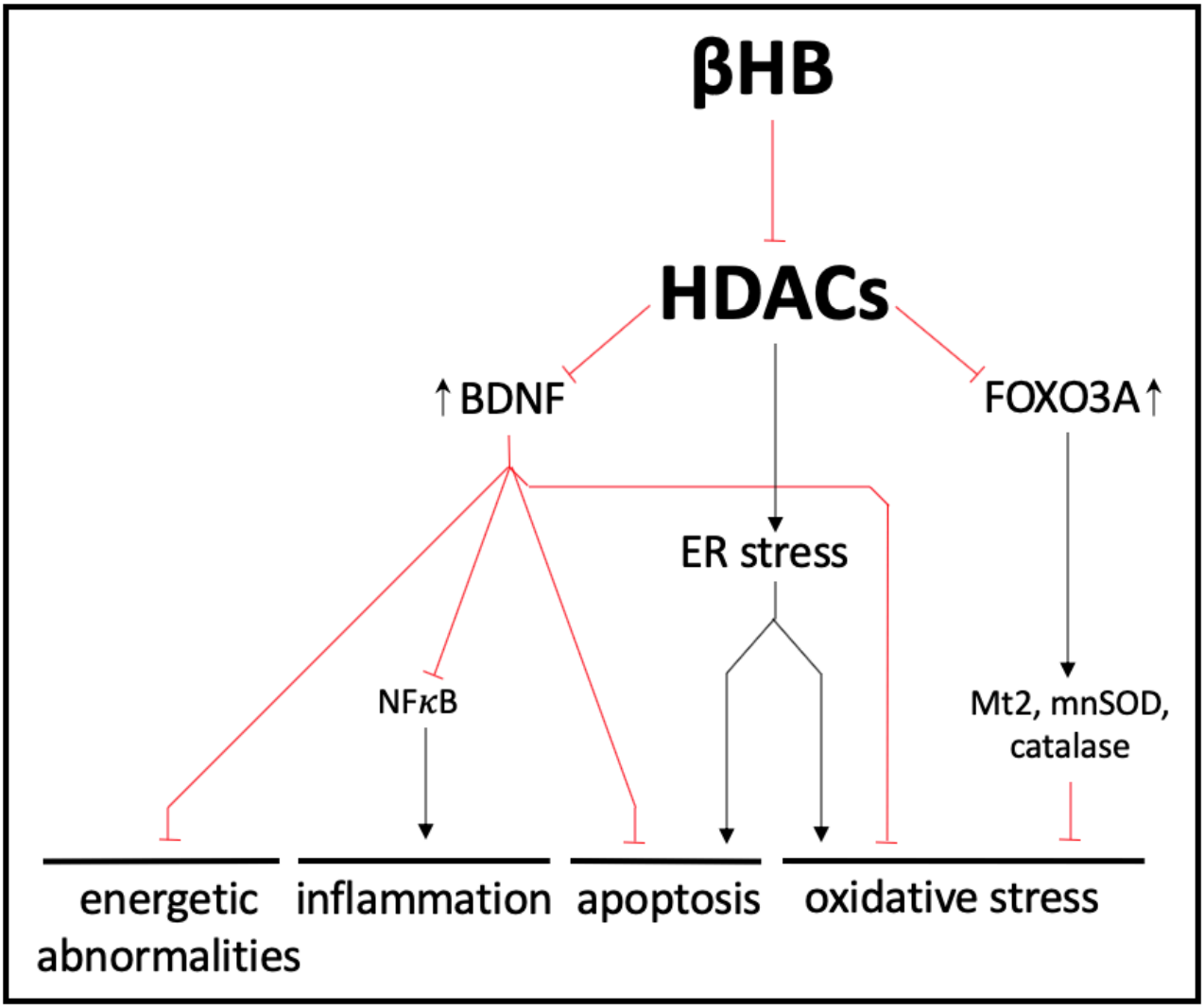


Figure 7.5 βHB exerts neuroprotective effects by inhibiting histone deacetylases (HDACs)

βHB-mediated HDAC inhibition increases BDNF and FOXO3A expression and prevents endoplasmic reticulum (ER) stress. In these ways, βHB protects neurons against energetic abnormalities, inflammation, apoptosis and oxidative stress.

7.1.8 Concluding remarks

Segregating the pathologies underlying PD is, to some extent, an arbitrary exercise because they are deeply interconnected. Energetic abnormalities cause cells, which have been unable to maintain homeostasis, and thus may have compromised genomes, to undergo apoptosis [196]. Apoptosis can deplete the extracellular pool of neuroprotective BDNF, thereby increasing inflammation [187, 188]. The NO produced as part of the inflammatory response can induce oxidative stress [177], which can cause mitochondrial damage and increase the energy crisis (Figure 7.6, outermost loop).

Of course, PD pathology is not a simple loop, but a complex network. Oxidative stress induces DNA damage, exciting the activity of the DNA repair protein, PARP1, which catabolizes the SIRT1 cofactor, NAD^+ [122], and thus precipitates further oxidative stress, energy crisis, and apoptosis. ROS also directly induces apoptosis and NFκB-mediated inflammation, the latter of which accentuates oxidative stress, causes apoptosis, and, because NFκB is induced by the cytokines that it induces, more inflammation [176] (Figure 7.6).

The pathologies that underlay PD are synergistic. This interconnectedness undermines any intervention aimed at a single point within the network. Perhaps that is why no true preventative treatments exist and why the current standard of care remains the largely palliative option of dopamine replacement therapy. However, there is a flipside to this complexity, for any molecule that can target many points within the network, as βHB can, may capitalize on the feedback loops, such that the multiple mechanisms by which it operates add up to more than the sum of their parts. In this review, we attempted to integrate the still largely independent bodies of literature on the

biochemical effects of βHB and on that pathological mechanisms of PD in order to present a model for how βHB could be used to improve the multiple cellular pathologies of PD. This model, in combination with preliminary data that show the ketogenic diet may be ameliorative in PD [37], suggests that exogenous βHB, now available as an FDA-approved sports drink, may have therapeutic potential for the prevention and/or treatment of PD. Future studies, including those currently being conducted, will reveal the authenticity of this potential.

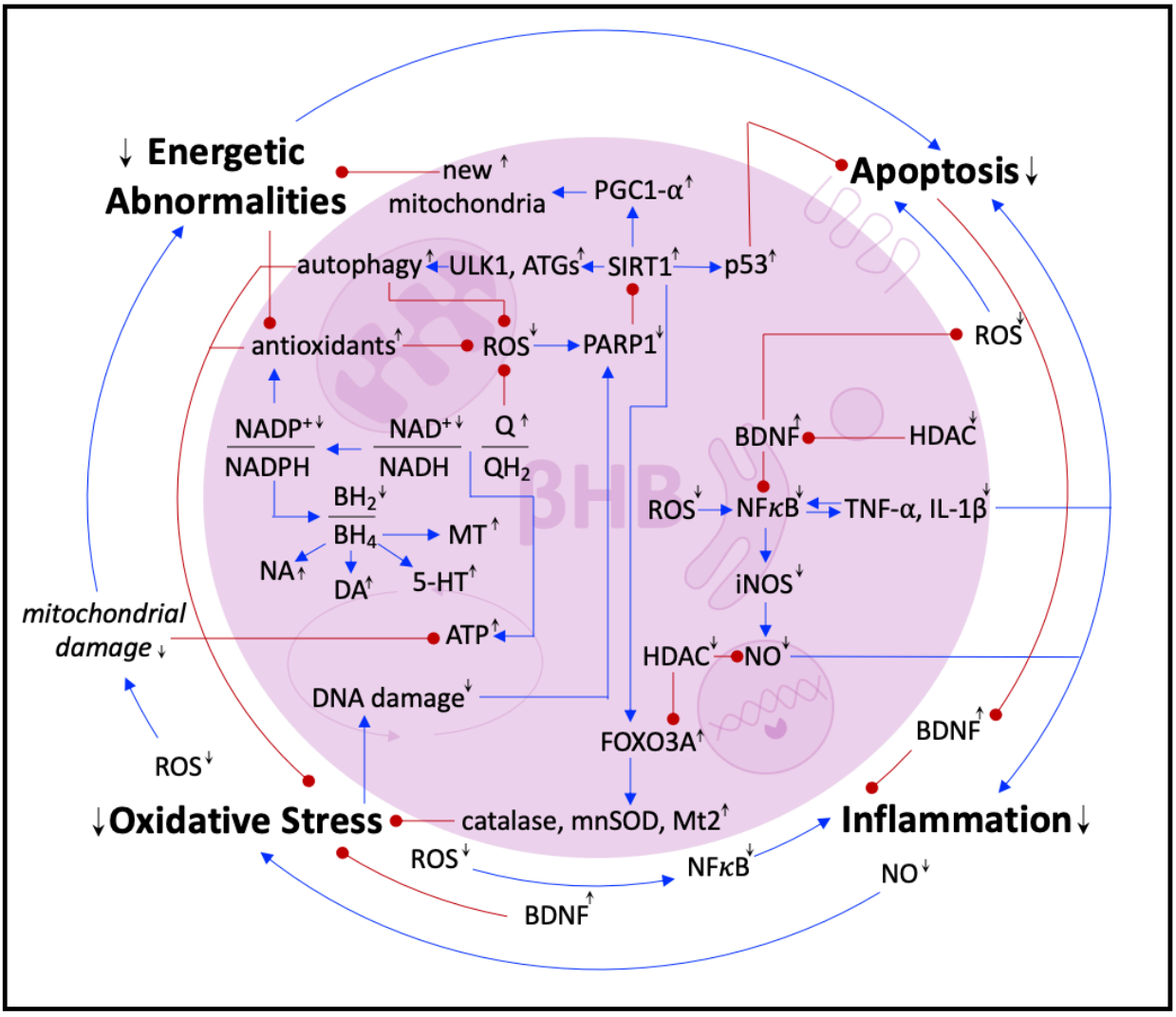


Figure 7.6 Summary of the interrelated pathologies of Parkinson' disease

Blue arrows represent activation or upregulation. Red dots represent inhibition or downregulation. Black up and down arrows represent the stimulatory or inhibitory effect of βHB on a given metabolite, ratio, protein or process.

7.2 **Norwitz NG**, Jaramillo JG, Clarke K, Mota AS. Ketotherapeutics for neurodegenerative disorders. *International Review of Neurobiology*. 2020

7.2.1 Statement of relevancy

This textbook chapter elaborates upon the themes presented in the introduction: neurodegenerative and neurological diseases are a function of metabolic disturbances – energetic abnormalities (mitochondrial dysfunction and insulin resistance), oxidative stress, and inflammation – and βHB, either produced endogenously or through exogenous supplements like the ketone ester (KE), has the potential to help correct these metabolic disturbances. In addition to generalizing the aforementioned themes beyond Parkinson's disease (PD) to include other neurodegenerative diseases, this work discusses the diverse array of strategies available to help patients enter ketosis (ketogenic diets, supplements, fasting).

7.2.2 Abstract

Alzheimer's disease and Parkinson's disease are, respectively, the most prevalent and fastest growing neurodegenerative disorders worldwide. The former is primarily characterized by memory loss and the latter by motor symptoms of tremor and bradykinesia. Both Alzheimer's disease and Parkinson's disease are progressive diseases that share several key underlying mitochondrial, inflammatory, and other metabolic pathologies. This review will detail how these pathologies intersect with ketone body metabolism and signaling, and how ketone bodies, particularly D-β-hydroxybutyrate, may serve as a potential adjunctive nutritional therapy for two of the world's most devastating conditions.

7.2.3 Mitochondrial dysfunction

7.2.3a Definition

"Mitochondrial dysfunction" is a blanket term used to describe all manner of maladaptive mitochondrial phenotypes, including impairment in electron transport chain components, fission and fusion dynamics, intracellular trafficking, mitophagy, and so on. However, the concept can be simplified by defining mitochondrial dysfunction in terms of its two ultimate consequences:

(1) Underproduction of ATP.

(2) Overproduction of reactive oxygen species (ROS).

For the purposes of this review, we will define mitochondrial dysfunction by these two consequences. In this section, we first present data that suggest mitochondrial dysfunction is a common, and perhaps causal, factor in the pathogenesis of Alzheimer's disease (AD) and Parkinson's disease (PD), before moving into a mechanistic discussion about how the ketone body, D-β-hydroxybutyrate (βHB), may correct or compensate for mitochondrial dysfunction.

7.2.3b Mitochondrial dysfunction in the pathogenesis of Alzheimer's disease and Parkinson's disease

Studies on cognitively normal individuals with a maternal family history of AD, carriers of the AD-risk allele, *ApoE4*, and animal models suggest that one of the earliest recognizable features of AD is a decrease in cerebral glucose metabolism (cGM) [197-200]. Although the exact cascade of pathological events remains to be determined, and may vary among individuals, evidence suggests that a decrease in cGM may coincide with, or even precede, the preclinical deposition of amyloid β (Aβ) [201, 202]. The AD-associated deficiency in cGM is attributable to impaired mitochondrial oxidative phosphorylation and is consistent with the observation that electron transport chain complex IV is less active than normal both in diagnosed AD patients and in individuals at high risk for developing AD [203, 204].

Mitochondrial dysfunction can contribute to the classical pathological hallmark of AD, Aβ plaques, by promoting the amyloidogenic processing of amyloid precursor protein (APP) [205]. Several lines of evidence support the hypothesis that mitochondrial dysfunction precedes Aβ pathology, including that complex IV inhibition induces the amyloidogenic pathway and that cell lines created by transferring mitochondrial DNA from AD patients into healthy cells exhibited a decrease in complex IV activity and ATP production with an increase in ROS [206, 207]. However, since Aβ oligomers (and also tau oligomers) can reciprocally induce mitochondrial dysfunction (by mechanisms that include directly impairing respiratory chain protein function, increasing mitochondrial membrane permeabilization, inducing mitochondrial fission, disrupting mitophagy, and impairing axonal transport [208], whether the amyloid and tau pathologies, or

mitochondrial dysfunction, occur first to initiate the putative positive feedback loop remains an open question (Figure 7.7).

Swerdlow et al. (2004) have proposed that, while dysfunctional amyloid processing may be the primary insult in the 5% of cases caused by deterministic genetic mutations, in the remaining 95% of sporadic AD cases, mitochondrial dysfunction represents the primary insult [209]. While it's important to note that very low levels of intracellular Aβ, even in the absence of extracellular Aβ deposition, can impair mitochondrial function [210], Swerdlow's "mitochondrial cascade hypothesis" has gained traction in the field. Perhaps the most human-relevant line of evidence supporting the hypothesis regards the maternal heritability of AD. Recall, complex IV is underactive in AD [203] and that mitochondria and mitochondrial DNA, which codes for electron transport chain components, including all three catalytic components of complex IV [211], are inherited by the embryo from the mother's egg. In this context, it is interesting that maternal inheritance impacts a person's risk of having a decreased cGM and developing AD far more than paternal inheritance [198, 212, 213]. Strikingly, cognitively normal adults who have mothers with AD exhibit a 50% decrease in complex IV activity compared to adults who have fathers with AD [204]. Such data are consistent with the notion that, at least in some cases, mitochondrial dysfunction may affect a person's chances of developing neurodegenerative disease as early as conception.

The PD brain is similarly characterized by a decrease in ATP levels [36] and a corresponding decrease in electron transport chain protein function; although, in PD, complex I activity is impaired [23]. In fact, animal models of PD often rely on toxins that inhibit complex I to induce

mitochondrial dysfunction and parkinsonism (MPTP and rotenone). Furthermore, not only are the environmental risk factors of PD associated with mitochondrial dysfunction, but all the major known risk genes for PD affect mitochondrial function, including *SNCA, LRRK2, VPS35, GBA, CHCHD2, PINK1, Parkin, DJ-1, PLA2G6, ATP13A2*, and *FBXO7* [214, 215].

Of particular interest is the protein product of *SNCA*, α-synuclein. As is the case for Aβ and tau in AD, α-synuclein in PD may induce, and be induced, by mitochondrial dysfunction [216] (Figure 7.7). Evidence suggests that this positive feedback loop may involve complex I inhibition and mitochondrial dysfunction contributing to a decrease in autophagy, the ATP-dependent cellular recycling process known to promote α-synuclein disposal [217, 218]. α-Synuclein, which itself contains a mitochondrial targeting sequence, can, in turn, impair mitochondrial protein import, alter mitochondrial morphology, induce oxidative stress, and even further inhibit complex I in order to establish a vicious cycle that culminates progressive neurodegeneration [161, 216].

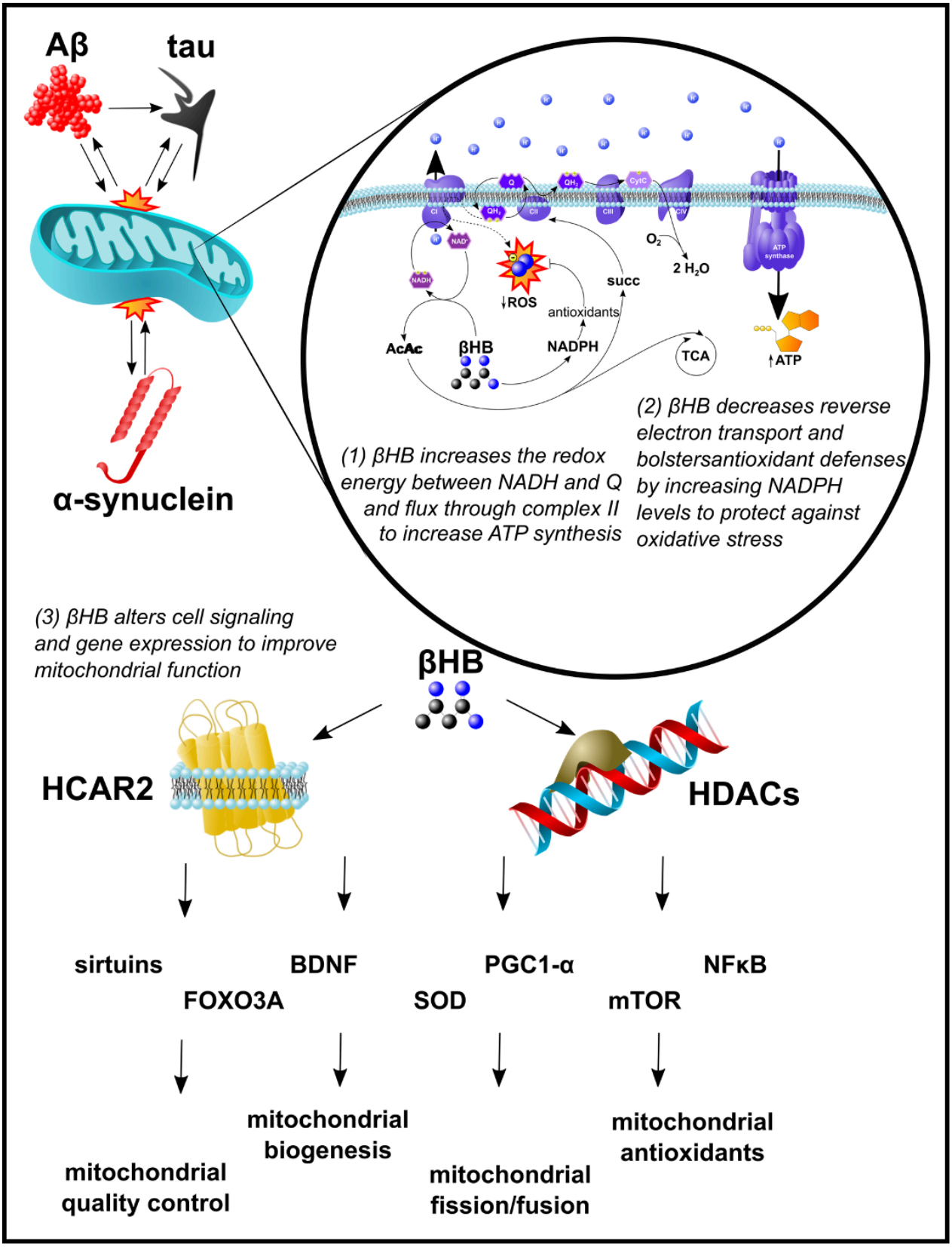

Aβ
tau
α-synuclein
(1) βHB increases the redox energy between NADH and Q and flux through complex II to increase ATP synthesis
(2) βHB decreases reverse electron transport and bolstersantioxidant defenses by increasing NADPH levels to protect against oxidative stress
(3) βHB alters cell signaling and gene expression to improve mitochondrial function
βHB
HCAR2
HDACs
sirtuins
FOXO3A
BDNF
PGC1-α
SOD
NFκB
mTOR
mitochondrial quality control
mitochondrial biogenesis
mitochondrial fission/fusion
mitochondrial antioxidants

Figure 7.7 Neurodegenerative pathology, mitochondrial dysfunction, and βHB

The amyloid (Aβ) and tau pathologies of Alzheimer's disease and the α-synuclein pathology of Parkinson's disease are in positive feedback with mitochondrial dysfunction. Mitochondrial dysfunction is characterized by (i) the underproduction of ATP and (ii) overproduction of reactive oxygen species (ROS). βHB can improve mitochondrial function through three mechanisms: (1) βHB catabolism decreases the NAD^+/NADH ratio and increases the Q/QH_2 ratio to increase the redox span of the electron transport chain and, thereby, increase the generation of ATP. In addition, the rate limiting step of βHB catabolism generates succinate, an oxidative fuel for complex II that bypasses the complex I blockade present in Parkinson's disease to further increase ATP production in this condition. (2) By increasing the Q/QH_2 ratio and levels of NADPH, βHB catabolism decreases the generation of ROS by reverse electron transport (dashed arrow) and bolsters antioxidant defences. (3) βHB is also a signaling molecule that binds to its own G-protein coupled hydroxycarboxylic acid receptor 2 (HCAR2) and inhibits histone deacetylases (HDACs), both widely expressed throughout the brain, to regulate a wide variety of critical enzymes, transcription factors, and cofactors and, thereby, improve mitochondrial function.

7.2.3c β-hydroxybutyrate may correct or compensate for mitochondrial dysfunction in Alzheimer's disease and Parkinson's disease

The first study investigating whether βHB can protect cell models of AD and PD was performed in by Kashiwaya and colleagues (2000). In this study, the investigators treated hippocampal neurons with neurotoxic Aβ and dopaminergic neurons with 1-methyl-4-phenylpyridinium (MPP^+) to model AD and PD, respectively. As expected, these treatments decreased neuron viability. However, when the cells were pretreated with a clinically achievable and safe concentration of βHB (4 mM), they were resistant to Aβ and MPP^+-induced cell death [90]. In this seminal publication, the authors postulated that the protective effects of βHB were due to improved mitochondrial function, a prediction that has been supported by other studies that have shown exogenously administered βHB can boost ATP production and/or prevent Aβ- or MPP^+-induced superoxide generation in neurons *in vitro* [81, 85, 219].

In vivo studies have also begun to explore the potential benefits of exogenous βHB on mitochondrial function in animal models of AD and PD. In one such study, a ketone monoester, an orally ingestible compound that is metabolized directly into βHB, was fed to 3xTgAD mice starting at 8 months of age for a duration of 8 months. When the brains of the 16-month old mice were examined, the ketone monoester supplemented mice exhibited an increase in the Gibb's free energy (ΔG) of ATP hydrolysis and a decrease in lipid and protein oxidation in their hippocampi relative to controls [220]. The same research group also showed that the ketone diet decreased hippocampal Aβ and p-tau load and improved anxiety and context-dependent memory in AD mice [221]. Complementary results have been obtained from a mouse model of PD in which subcutaneously administered exogenous βHB appeared to help circumvent the PD-associated

complex I blockade (by increasing electron entry at complex II of the electron transport chain), increase ATP production, and improve symptoms of parkinsonism [81].

These exciting preliminary findings beg the question, how might βHB improve mitochondrial function in AD and PD? Based on existing literature, the mechanisms by which βHB, either exogenously administered or endogenously produced, may increase mitochondrial ATP production and decrease mitochondrial ROS in the brain can be divided into three categories, each of which is elaborated upon in the following three paragraphs: (1) Effects on redox ratios and electron transport chain function, (2) effects on ROS production, nicotinamide adenine dinucleotide phosphate (NADPH), and antioxidant status, and (3) effects on cell signaling and gene expression.

(1) The effects on redox ratios and electron transport chain function refers, first and foremost, to the positive effect of βHB catabolism on the redox span between the nicotinamide adenine dinucleotide (NAD^+/NADH) and coenzyme Q (Q/QH_2) couples. In oxidative metabolism, NADH passes its electron pair through complex I to ubiquinone (Q) to generate NAD^+ and ubiquinol (QH_2). Since the NAD^+/NADH couple has a more negative redox potential (i.e., holds electrons in a higher energy state) than the Q/QH_2 couple, this process of passing electrons from NADH to Q liberates potential energy that is used to pump protons from the matrix into the intermembrane space. Interestingly, βHB catabolism decreases the matrix NAD^+/NADH ratio while increasing the Q/QH_2 ratio (at least in βHB-perfused rat hearts), increasing the difference in redox potentials between these two couples [80]. The effect of increasing the "redox span" between electron carriers is analogous to increasing the height span from which a bowling ball is dropped to the

ground. In both cases, more energy is available to do work. In the case of the bowling ball height span, more kinetic energy is available to break your toes. In the case of the NAD^+/NADH-Q/QH_2 redox span, more electrons can be pumped across the inner mitochondrial membrane to fuel ATP production by chemiosmosis. Moreover, βHB catabolism may be able to increase ATP production in the PD brain by circumventing the PD-associated blockade of complex I [72, 160, 161]. This mechanism makes biochemical sense because the rate limiting step of βHB catabolism generates succinate, an oxidative fuel that feeds into complex II, and, thereby, should bypass the complex I blockade. This more PD-specific mechanism is supported by *in vivo* data showing that βHB protected PD mice from neurodegeneration, but not when flux through complex II was blocked [81]. In summary, by increasing the redox span between NAD^+/NADH and Q/QH_2, and by increasing flux through complex II in PD, βHB catabolism may increase the production of ATP, alleviating one of the two ultimate consequences of mitochondrial dysfunction (Figure 7.7).

The other consequence of mitochondrial dysfunction, oxidative stress, may be addressed by the effects of βHB on ROS production, NADPH, and antioxidant status. As mentioned in the previous paragraph, βHB catabolism increases the Q/QH_2 ratio. In addition to increasing the redox span within the electron transport chain to increase proton pumping and ATP production, a higher Q/QH_2 ratio also carries the benefit of a decrease in "reverse electron transport", the process by which most ROS are generated by mitochondria. In reverse electron transport, QH_2, rather than passing electrons forward to complex III, passes electrons backwards at complex I to oxygen to generate superoxide radicals. Thus, by increasing the Q/QH_2 ratio, βHB catabolism decreases the generation of ROS. Complimentarily, βHB catabolism can also bolster antioxidant defences by decreasing the $NADP^+$/NADPH ratio [30, 109]. Unlike NADH, which functions to support

oxidative metabolism, NADPH is used for the reductive biosynthesis of antioxidants. In fact, NADPH is required to support all known intracellular antioxidant species, including glutathione, thioredoxins, and vitamins C and E [109]. The multiple mechanisms by which βHB catabolism can increase NADPH levels and antioxidant defences in neurodegenerative diseases has been reviewed elsewhere [30] (Figure 7.7).

The final mechanism by which βHB could alleviate mitochondrial dysfunction in AD and PD is the most expansive. βHB is not only a nutritional macromolecule, but also a signaling molecule. It has several G-protein coupled receptors, including hydroxycarboxylic acid receptor 2 (HCAR2), and inhibits class I/II histone deacetylases (HDACs) to alter gene expression [108, 162, 184]. A detailed discussion of the multitudinous effects of βHB signaling on cell metabolism is beyond the scope of this chapter, but may include induction of the pro-longevity sirtuin proteins and Forkhead box O 3A (FOXO3A) transcription factor, the neurotropic factor brain-derived neurotrophic factor (BDNF), the antioxidant enzyme manganese superoxide dismutase (MnSOD), the master regulator of mitochondrial biogenesis PPARG coactivator 1 alpha (PGC1-α), and several autophagy proteins, as well as inhibition of the anti-longevity mechanistic target of rapamycin (mTOR) and pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [30]. Either directly or indirectly, any or all of such cell signaling effects could improve mitochondrial quality, increase mitochondrial ATP production, and decrease oxidative stress (Figure 7.7).

7.2.4 Inflammation

7.2.4a Central role in neurodegenerative conditions

It is generally accepted that neuroinflammation contributes to neurodegenerative diseases. This neuroinflammation is mediated, in large part, by hyperactive microglia [222] and astrocytes [223], which together contribute to chronic low-grade release of cytokines, such as the interleukins, IL-1β and IL-6, and tumor necrosis factor α (TNFα), pathological levels of phagocytosis, disease-specific toxin production, and neurological damage in general [224-226].

7.2.4b Microglia activation

Microglia activation leads to the release of inflammatory factors, such as nitric oxide (NO) and prostaglandins [225]. These responses are highly controlled and are accompanied by a metabolic shift that remains poorly understood. In neurodegenerative diseases, the increased signaling caused by neuronal damage and impaired circuits in different brain regions cause dysregulation of microglia homeostasis, further increasing release of proinflammatory cytokines that damage neurons [227].

In AD, the accumulation of Aβ activates microglia via cluster of differentiation 36 (CD36) and the Toll-like receptor (TLR) heterodimer TLR2-TLR6. In turn, microglial activation and secretion of NO may contribute to the formation of senile Aβ plaques [228], establishing a positive feedback loop. Moreover, the contribution of hyperreactive microglia to the formation of tau tangles may be a missing link in the amyloid cascade model of AD in which Aβ pathology leads to downstream tau pathology [225].

In PD, higher expression of human leukocyte antigen-DR isotype (HLA-DR), a major histocompatibility complex (MHC) class II receptor, has been found in the post-mortem substantia nigra [229, 230]. This, together with the fact that α-synuclein activates TLRs [231, 232], is consistent with a critical role for inflammation in the death of dopaminergic neurons [233].

7.2.4c Astrocyte activation

Astrocytes have a broad set of functions, including regulating blood-brain barrier permeability and maintaining synaptic integrity. Importantly, astrocytes have a metabolic role that includes quenching inflammatory factors [234].

Activated astrocytes are divided into two subgroups: A1 astrocytes are, to generalize, neurotoxic [235], whereas A2 astrocytes are neuroprotective [223, 236, 237]. Although an imbalance in the equilibrium towards the harmful A1 fate is presumed in cases of neurodegenerative disease, it is important to treat this matter with nuance and give credit to activated astrocytes as more than the "bad guys." For example, transplantation of astrocytes helps to clear Aβ plaques [238]. However, astrocytes can also produce neurotoxic Aβ oligomers [239]. Furthermore, Aβ might disrupt astrocyte metabolism, possibly contributing to an increase in the (neurotoxic) A1 over the (neuroprotective) A2 fate, inducing yet another positive feedback loop [240]. In PD, the situation is similar: early accumulation of α-synuclein in astrocytes causes an increase in microglia hyperactivation [241], blood-brain barrier permeability, and energy imbalances [223], all of which are associated with disease progression.

7.2.4d β-hydroxybutyrate dampens neuroinflammation via histone deacetylase and NLRP3 inflammasome inhibition

Ketogenic diets have proved successful in the treatment of multiple neurodegenerative disorders [37, 242], although it is not entirely clear whether the benefits are derived from the ketones themselves or other aspects of the diet. Still, by acting as potent signaling molecules to alter intracellular signaling cascades and gene expression, there is a high probability that at least some of the benefits derive from βHB itself [243, 244].

Among other signaling functions, βHB inhibits HDACs [245], and it is possible that, in this way, βHB calms microglia and astrocyte hyperactivation. For example, βHB suppresses HDAC-induced oxidative stress [191] and induces microglia to adopt the anti-inflammatory M2 morphology [246].

Additionally, one of the key regulators of inflammation is the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [247]. Interestingly, while βHB has been shown to inhibit the NLRP3 inflammasome, this effect seems to be independent of HDAC inhibition and other better-known βHB signaling mechanisms; rather, βHB inhibits the inflammasome by altering potassium flux [248] (Figure 7.8).

Evidently, there is much more clinical and basic science work to be done to elucidate the putative cell-signaling-dependent anti-inflammatory effects of βHB.

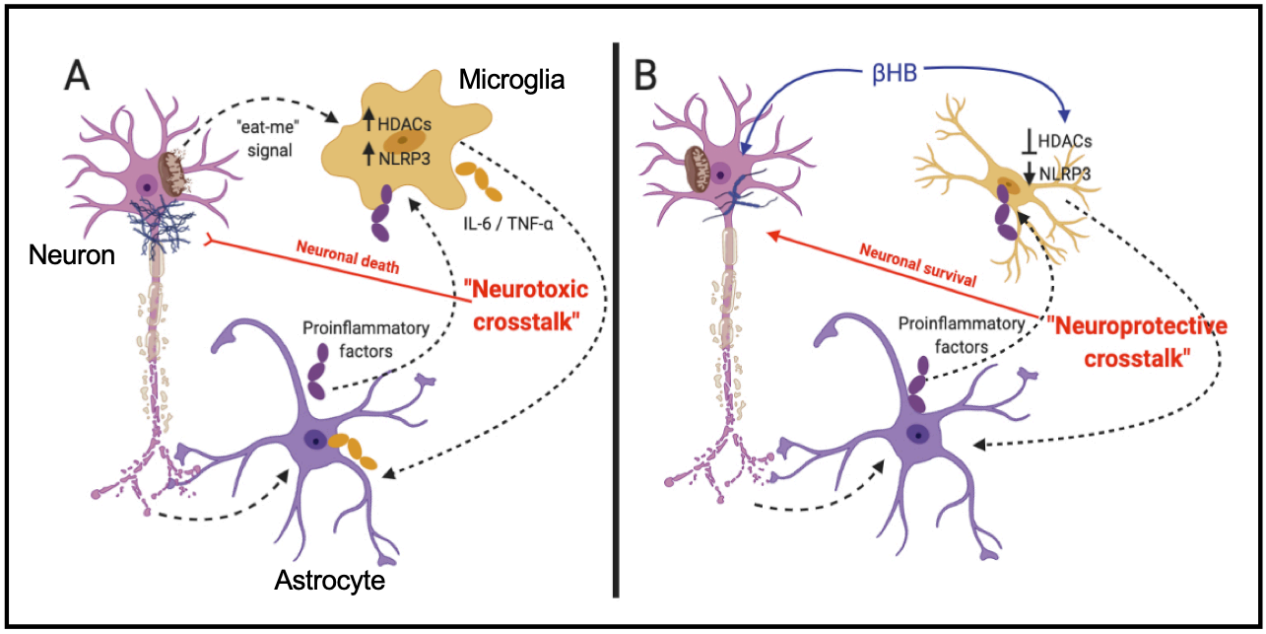


Figure 7.8 Anti-inflammatory role of βHB in neurodegenerative disorders

(A) Neurodegeneration induces the A1 proinflammatory astrocyte (purple cell) response and triggers microglia-mediated (orange cell) phagocytosis. Transcription and release of proinflammatory factors increase the HDAC- and NLRP3-mediated inflammatory responses in glia, promoting neuronal (pink cell) death by neurotoxic crosstalk between proinflammatory microglia and astrocytes. (B) βHB may decrease inflammation signaling, in part, by inhibiting HDACs and impairing NLRP3 inflammasome formation. (Figure composed by Jaramillo JG.)

7.2.5 Glucose and insulin

7.2.5a Lack of energy substrates in neurodegeneration

For its size, the brain is our most energetically expensive organ [249]; however, it is metabolically inflexible. Under non-ketotic conditions, human brains, which cannot metabolize fat as fuel, are dependent on glucose [250]; and, correspondingly, deficiencies in the brain's ability to metabolize glucose are thought to contribute to neurodegeneration.

Even in the preclinical stages, patients with AD [251] and PD [252] exhibit impaired cGM but not impaired cerebral uptake of ketones [253]. As it is believed that our species developed its superior capacity for ketogenesis specifically to fuel the brain in times of glucose scarcity, in our perspective, there are no apparent compelling reasons that ketones would not prove as beneficial in the context of pathological metabolic scarcity as they evidently were in supporting our species' evolutionary exposure to glucose dietary scarcity.

7.2.5b Insulin resistance: a hallmark of Alzheimer's disease

Insulin is a crucial regulator of many neuronal processes [254]. Both the presynaptic axon terminal and postsynaptic density are highly enriched with insulin receptors. In these compartments, insulin modulates catecholamine release and uptake, the trafficking of ion-gated channels, and the expression and localization of neurotransmitter receptors, such as GABA and NMDA receptors [255] (Figure 7.9i).

Interestingly, brain insulin resistance can occur without systemic insulin resistance and, because insulin reaches the cerebrospinal fluid (CSF) via the capillary endothelial cells of the blood-brain barrier and its transport and elimination are regulated separately [256], brain cells can be exposed to different insulin levels than those of peripheral tissues.

While it is true that insulin resistance increases with age [257], it is worth observing that inducing localized insulin resistance mimics many of the biochemical and clinical features of AD [258] and that selective insulin transport through the blood-brain barrier is upregulated in many conditions that are prone to neurodegeneration, including obesity, diabetes, hypertriglyceridemia, and chronic inflammation [259].

At a cellular level, insulin resistance can result from a decrease in glucose transporters in the membrane [251, 260, 261] and inhibition of insulin signaling proteins, such as protein kinase B (AKT), or their regulatory partners, such as the canonical WNT-β-catenin-Glycogen synthase kinase 3β (GSK3β) pathway [262, 263] (Figure 7.9ii-iv). Correspondingly, direct pharmacological AKT activation rescues AD-like memory impairment and aberrant synaptic plasticity [264].

Insulin resistance and associated hyperglycemia, enhance other pathological hallmarks in many neurodegenerative diseases, including AD. The glycation of Aβ oligomers increase their pathogenicity [265]. In addition, insulin degrading enzyme (IDE) degrades both insulin and Aβ. Therefore, hyperinsulinemia prevents Aβ degradation and, reciprocally, Aβ can further exacerbate insulin resistance by preventing insulin degradation (Figure 7.9v) [266-269]. What's more, such vicious cycles can compound: For example, insulin-AKT pathway dysfunction can contribute to

an increase in GSK3β activity, which is antagonistic to neuroprotective WNT signaling [270, 271] (Figure 7.9iv), whereas GSK3β can contribute to insulin resistance by phosphorylating and inhibiting insulin receptor substrate 1 (IRS1) [272] (Figure 7.9vi). Thus, insulin resistance can exacerbate AD via a complex network of pathological positive feedback interactions [273].

In PD, insulin resistance impairs nigrostriatal dopamine function [274], α-synuclein negatively regulates insulin signaling [275], and insulin receptors in the substantia nigra are decreased [276].

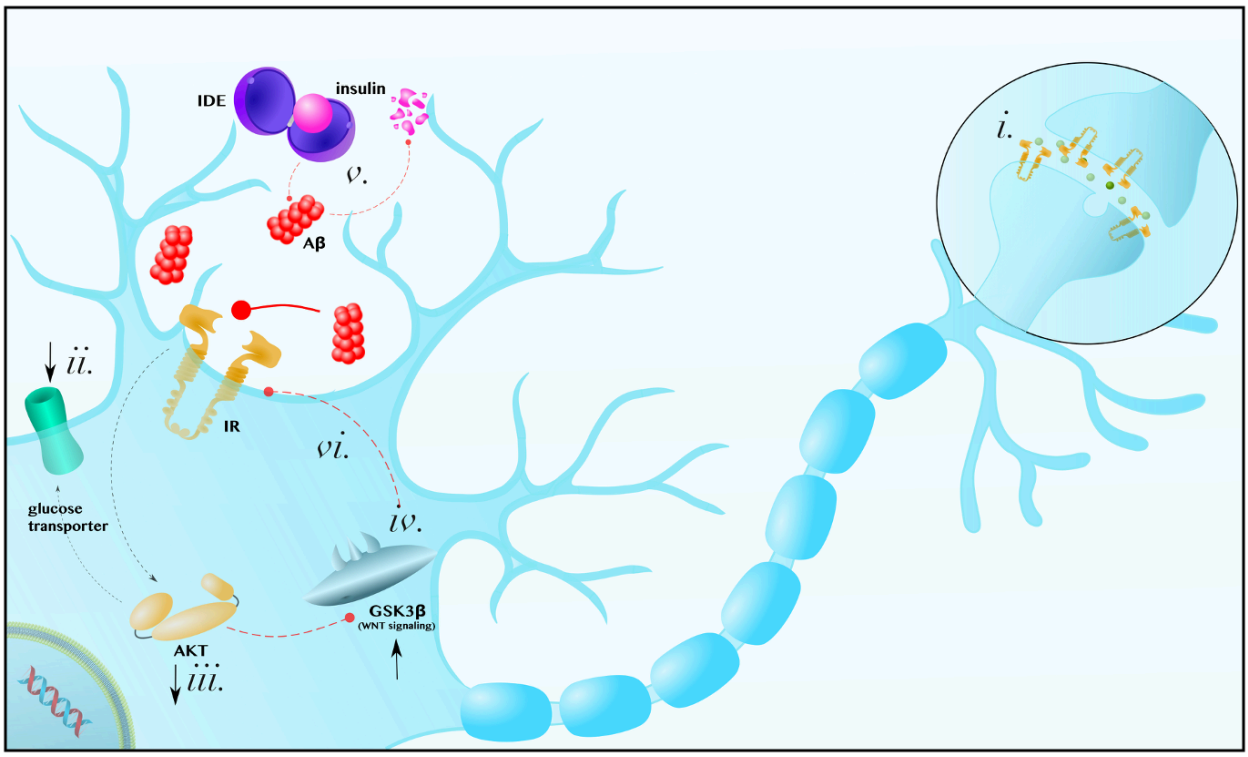


Figure 7.9 Insulin in the brain

(i) Insulin receptors (IR) are enriched at synapses, where they regulate neurotransmitter release and the localization of receptors. (ii) Insulin resistance decreases glucose transporters in the membrane, (iii) leads to the inhibition of downstream proteins, like AKT, (iv) and induces neurotoxic anti-WNT GSK3β activity. (v) Hyperinsulinemia prevents Aβ degradation by insulin degrading enzyme (IDE), establishing a positive feedback loop. (vi) Furthermore, GSK3β activity, which is antagonistic to neuroprotective WNT signaling, also inhibits insulin signaling. The figure is meant to be representative, not comprehensive, regarding the mechanisms by which insulin resistance can contribute to the degeneration of neurons.

7.2.6 Ketotherapeutics

Because brain cells can oxidize ketone bodies and ketones regulate fuel metabolism [277], interventions that induce ketosis offer an exciting opportunity to prevent, slow, halt, or reverse the progression of neurodegenerative diseases [30]. Additionally, most ketogenic interventions are safe [55, 278, 279], well tolerated, and improve other exacerbating comorbidities, such as type II diabetes [280, 281] and inflammation [282].

7.2.6a Endogenous nutritional ketosis

In monitored patients, fasting has proven to be safe [283], a statement also supported by the fact that, for centuries, billions of people have fasted safely for religious reasons. The hormonal profile of the fasting state is perhaps the largest difference between endogenous and exogenous ketosis: endogenous ketosis is marked by a low-insulin, high-cortisol and glucagon environment that heavily promotes lipolysis [284].

High-fat low-carbohydrate ketogenic diets also result in a low-insulin, high-cortisol and glucagon environment, but differ from fasting in that ketones are derived partly from dietary fat, as well as from body fat. Foods high in medium chain triglycerides are particularly ketogenic compared with other fat sources [285].

7.2.6b Exogenous nutritional ketosis

Many ketone salts are commercially available, their main limitation being that unhealthy intake of salt is required to achieve therapeutic levels of βHB. In addition, most salts provide a racemixture, rather than the pure bio-relevant D-βHB isoform [58].

By contrast ketone esters, specifically the best-studied βHB monoester, induces deep ketosis (levels observed after several days of fasting or following a strict ketogenic diet; >3.0 mM) because it yields only D-βHB and does not carry the restriction of the accompanying salt bolus. Furthermore, ketone esters allow for the accurate titration of blood ketone levels within 30 minutes [87]. It is important to note that ketone salts and esters have different metabolic [58] and tolerability profiles [286].

To date, there is anecdotal support for the use of ketone esters in human AD [287] and clinical studies are in progress for their use in PD. For example, our group, Norwitz et al., is currently investigating whether this form of exogenous ketone can increase cerebral energy production (as measured by magnetic resonance spectroscopy) improve physical performance, and improve broad-spectrum symptomology and quality of life in persons with PD [288, 289].

7.2.6c Endogenous versus exogenous nutritional ketosis

Endogenous and exogenous ketosis each comes with its own potential benefits and drawbacks. Endogenous ketosis, by requiring a more comprehensive metabolic shift, has the advantage of activating a larger array of metabolic pathways. Furthermore, fasting and ketogenic diets may help the body adapt to better utilize ketones as a fuel and signaling molecule, as opposed to exogenous

ketogenic strategies in which the body can choose to continue to metabolize glucose. Finally, whereas it is well established that fasting is safe in the long-term, and the same can also probably be said of well-formulated ketogenic diets, little is known about the long-term impact of the high-glucose, high-insulin, high-ketone condition that results from addition of a ketone supplement to a typical carbohydrate-rich Western diet. The latter is not a metabolic state to which our species evolved and it is possible that long-term exposure to simultaneously high glucose and ketones could have unforeseen consequences.

On the other hand, endogenous ketosis does not permit the specific targeting of ketone levels, whereas exogenous ketosis does. In particular, ketone esters permit the induction of deep ketosis (>3.0 mM), which may have particular therapeutic benefits by activation particular genetic and/or metabolic pathways. Furthermore, given the current state of food culture, social climate, and nutritional guidelines/common knowledge, fasting and ketogenic diets can come with compliance difficulties for many patients. This will, hopefully, change as culture and nutrition science evolve, but, at this time, imposes a serious practical limitation on endogenous ketosis interventions. Exogenous ketone supplements, by contrast, are easy to consume on a long-term basis. Finally, it is worth noting that ketone supplements can be stacked on top of fasting or ketogenic diets to induce deeper therapeutic ketosis without incurring the risk of long-term exposure to high glucose and ketones.

7.2.6d Ketogenic interventions in patients with mild cognitive impairment

Recently, there have been several ketotherapeutic interventions conducted in patients with mild cognitive impairment (MCI), a precursor to Alzheimer's disease. For example, in a randomized crossover pilot study of a Mediterranean-style ketogenic diet for MCI, a 6-week ketogenic diet improved Alzheimer's biomarkers, including CSF levels of Aβ and tau, as compared to a 6-week American Heart Association diet control [290]. Furthermore, medium chain triglycerides, which induce mild ketosis [285] have demonstrated clinical efficacy in several trials. In a 6-month study of 52 MCI patients, medium chain triglyceride consumption at 2 Tbsp/day improved episodic memory, executive function, and processing speed compared to baseline and compared to a placebo control [291]. A recent meta-analysis confirmed that medium chain triglyceride interventions in Alzheimer's patients tend to improve functional cognitive measures, in part, by inducing ketosis [292]. The general efficacy of these early studies may be due to the fact that, while neurodegenerating brains appear to lose their ability to metabolize glucose, ketones remain a viable fuel and cerebral ketone uptake tends to parallel blood ketone levels [293]. Therefore, it will be important for future studies implementing other interventions to investigate whether deeper levels of ketosis, such as those induced by ketone esters, are even more effective.

7.2.6e Ketogenic interventions, mitochondria, and SIRT3

Since this review placed particular emphasis on mitochondrial dysfunction as the basis for neurodegenerative disease, it is important to remark on the recent evidence implicating activation of mitochondrial sirtuin 3 (SIRT3), a protein whose activity is reduced in AD patients in association with Aβ pathology [294], as one mechanism by which ketogenic interventions may protect against AD. In AD mice, intermittent fasting to induce ketosis increased SIRT3 activity

and protected against hyperexcitability and hippocampal synaptic dysfunction [295]. While one could reasonably postulate that mechanisms related to intermittent fasting, other than ketosis, are responsible for the increase in SIRT3 activity, recent evidence suggests the neuroprotective effect is, indeed, due to βHB itself. In particular, supplementation with a ketone ester increased SIRT3 expression in a SIRT3 haploinsufficient mouse model of AD, preventing GABA neuron degeneration and protecting against excitotoxicity [294]. Therefore, the SIRT3 promoting anti-excitotoxicity effects ketones is one mechanism by which both endogenous and exogenous ketogenic interventions may prove therapeutic in neurodegenerative disease patients.

7.2.7 A note on intermittent fasting, metabolic switching, and disease prevention

It would be inappropriate to extrapolate from current clinical data that particular ketogenic interventions, while helping improve symptomology and disease markers in individuals already afflicted with neurodegenerative disease, would also help to prevent disease onset in individuals at risk for AD and PD. Given the growing popularity of ketogenic diets for brain health, even among individuals as young as their twenties, it is worth noting that no long-term trials on ketogenic interventions for complete neurocognitive disease prevention have yet been performed and, therefore, worth considering that promoting "metabolic flexibility" (in brains that still retain the ability to adequately utilize glucose) may be ideal for disease prevention. For example, intermittent fasting strategies that induce ketosis but also permit the body to switch on glucose metabolism in a cyclic manner can both activate ketosis-associated cellular repair and defence pathways, while also optimally promoting healthy anabolic processes, such as the growth of synapses, during periods of carbohydrate feeding [296, 297]. In the literature, this is commonly referred to as activating the "metabolic switch." While there is no data yet comparing the safety or

efficacy of chronic ketosis versus "metabolic switching" for neurodegenerative disease progression, it is logical to assume that the latter may come with certain to-be-discovered advantages for the simple reason that our species evolved to intermittent fast, rather than to chronically eat ketogenic diets or consume ketone supplements [297]. At this point, to argue for the superiority of ketogenic diets, ketone supplements, or intermittent fasting remains in the realm of speculation.

7.2.8 Summary

Most neurodegenerative diseases, including AD and PD, are associated with the key pathologies of mitochondrial dysfunction, neuroinflammation, and glucose hypometabolism and/or insulin resistance. Currently, there are no effective therapies for slowing the progression of either AD or PD. By addressing these core pathologies (and likely others), endogenously or exogenously induced ketosis might prove to be a novel and useful adjunctive therapeutic for these neurodegenerative conditions.

7.3 **Norwitz NG**, Mota AS, Norwitz SG, Clarke K. Multi-loop model of Alzheimer disease: an integrated perspective on the Wnt/GSK3β, α-synuclein, and type 3 diabetes hypotheses. *Frontiers in Aging Neuroscience*. 2019

7.3.1 Statement of relevancy

The clinical diagnoses of AD and PD create the impression that these are categorically distinct diseases with mutually exclusive pathologies. For example, amyloid and tau are the "Alzheimer's" proteins, whereas α-synuclein is the "Parkinson's" protein. In reality, the pathologies of AD and PD overlap. Amyloid, tau, and α-synuclein all interact, also in association with the underappreciated Wnt-signaling pathway and cerebral insulin resistance, which play important roles in both disorders.

This perspectives paper takes novel integrated molecular perspective on the two most common neurodegenerative conditions, concisely summarized in the culminating Figure 7.13. It resonates with the overarching theme of this thesis that, in order to generate tools sufficient to treat neurodegenerative and neurological diseases, the biomedical community should envision these disorders as networks of cellular dysfunctions that might only be treated by addressing multiple nodes in the network. In other words, neurodegenerative and neurological diseases are metabolic diseases that invite complementary metabolic therapies.

7.3.2 Abstract

As the prevalence of Alzheimer's disease continues to rise unabated, new models have been put forth to improve our understanding of this devastating condition. Although individual models may have their merits, integrated models may prove more valuable. Indeed, the reliable failures of monotherapies for Alzheimer's disease, and the ensuing surrender of major drug companies, suggests that an integrated perspective may be necessary if we are to invent multifaceted treatments that could ultimately prove more successful. In this review article, we discuss the Wnt/Glycogen Synthase Kinase 3β, α-synuclein, and type 3 diabetes hypotheses of Alzheimer's disease, and their deep interconnection, in order to foster the integrative thinking that may be required to reach a solution for the coming neurological epidemic.

7.3.3 Introduction

Alzheimer disease (AD) is among the most ominous of modern health epidemics. The current costs, both human and financial, are staggering and climbing at a precipitous rate. In the United States alone, 5.5 million adults live with AD, imposing an economic burden of $259 billion [298]. Over the next three decades, the number of people living with AD is expected to triple to 13.8 million and the economic costs are projected to quadruple to $1.1 trillion, single-handedly crippling the United States health care system. AD is also the only disease on the list of the top 10 disease causes of death for which there is currently no effective treatment [298].

AD is not alone in its ascent. Other chronic diseases, particularly Parkinson's disease (PD), a neurodegenerative disorder associated with the build-up of α-synuclein protein and death of dopaminergic neurons, and type 2 diabetes mellitus (T2DM) are increasing in prevalence at similarly alarming rates [299, 300]. Although AD, PD, and T2DM share common risk factors, chief among these being age, there is more to their relationship. Evidence suggests that the mechanisms underlying AD, PD, and T2DM interact synergistically [301-308].

In addition to the well-known amyloid cascade hypothesis of AD, other hypotheses have been proposed that include: (1) the Wnt/Glycogen Synthase Kinase 3β (GSK3β) hypothesis [309-311], (2) the α-synuclein hypothesis [308, 312], and (3) the type 3 diabetes hypothesis [302]. In this review article, we focus on the Wnt/GSK3β hypothesis, describing how it serves as a platform for a set of positive feedback loops that contribute to the pathogenesis of AD. In turn, we also discuss the α-synuclein and type 3 diabetes hypotheses, describing how they each constitute their own feedback loops and interact with the Wnt/GSK3β model.

7.3.4 Wnt/GSK3β

7.3.4a Overview of Wnt-signaling

Wnt-signaling refers to a set of highly conserved signal transduction pathways that are widely expressed throughout the body and that play a vital role both in neuronal development and in the maintenance of proper neuronal function in the adult human brain [313-316]. In this article, we focus on the better-studied canonical Wnt-β-catenin-signaling pathway, leaving the topic of the two non-canonical Wnt-signaling pathways (the Wnt-planar cell polarity and Wnt-calcium pathways) for others to discuss in depth [305, 314, 315, 317, 318]. Canonical Wnt-β-catenin-signaling (hereafter, referred to simply as Wnt- signaling) is initiated by the binding of Wnt ligands to the Wnt receptor pair, Low-Density Lipoprotein Receptor-Related Protein 6-Frizzled (LRP6-Fz). LRP6 then recruits Dishevelled (DVL), a scaffolding protein that sequesters GSK3β from the cytoplasm. The inhibition of GSK3β, a constitutively active kinase that targets the transcriptional cofactor β-catenin for proteasomal degradation, is central to Wnt-signaling. Simply put, Wnt-signaling inhibits GSK3β, permitting β-catenin to accumulate in the cytoplasm and translocate into the nucleus to mediate the transcription of genes, such as *BACE1* and *ADAM10* (elaborated upon below), involved in the pathogenesis of AD [315] (Figure 7.10A).

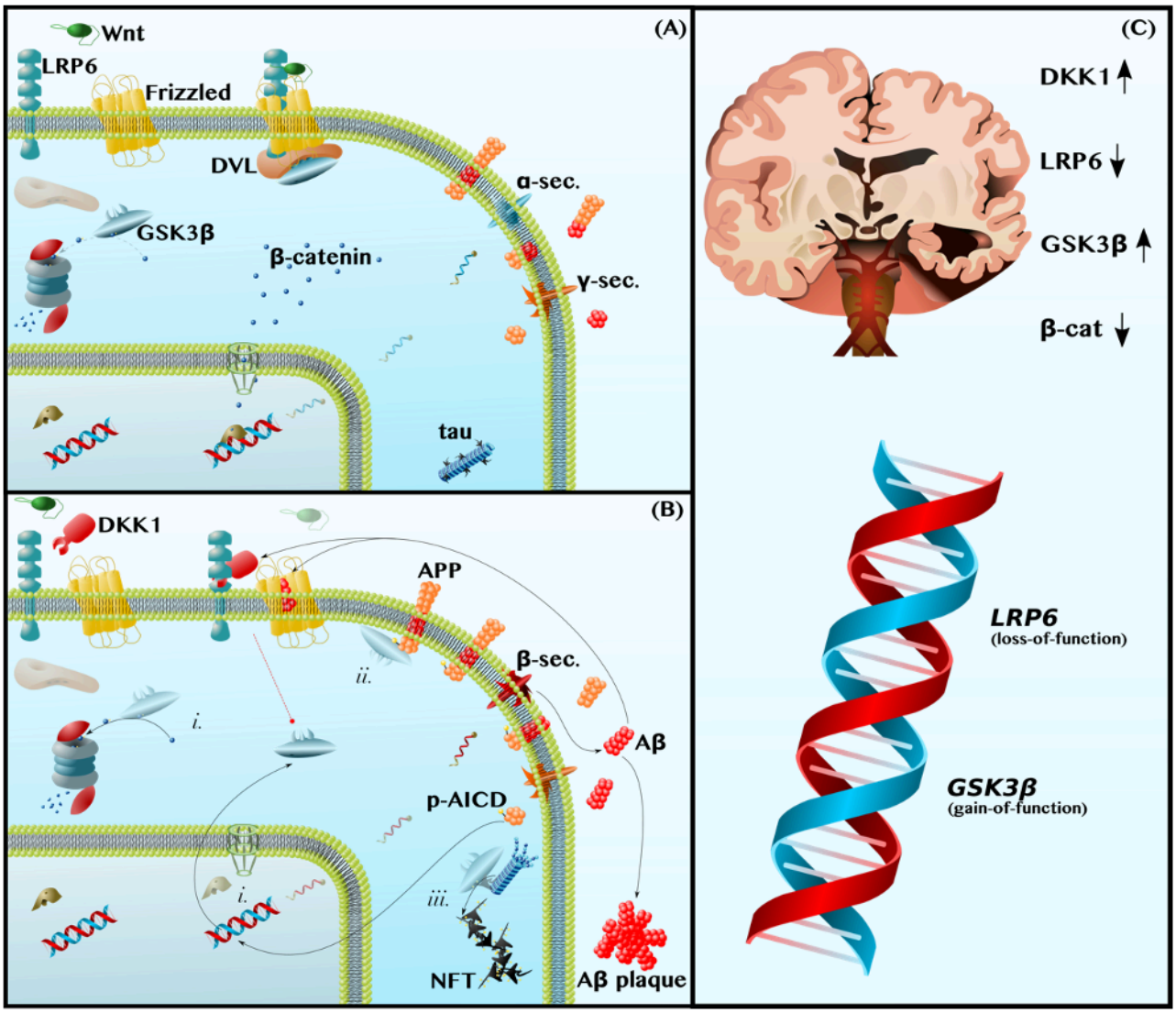

(A)
Wnt
LRP6
Frizzled
DVL
GSK3β
β-catenin
α-sec.
γ-sec.
tau
(B)
DKK1
APP
β-sec.
Aβ
p-AICD
NFT
Aβ plaque
i.
ii.
iii.
(C)
DKK1
LRP6
GSK3β
β-cat
LRP6
(loss-of-function)
GSK3β
(gain-of-function)

Figure 7.10 Dysfunctions in canonical Wnt-Signaling contribute to the neuropathology of Alzheimer disease

(A) Functional Wnt and Nonamyloidogenic Processing—Glycogen Synthase Kinase 3 β (GSK3β) is a constitutively active kinase that phosphorylates and targets β-catenin for proteasomal degradation. The binding of Wnt ligands to LDL Receptor-Related Protein 6 (LRP6) and Frizzled induces the receptor pair to bind Dishevelled (DVL), a protein that serves as a docking platform for GSK3β. The sequestration of GSK3β by the Wnt receptor complex permits β-catenin to accumulate and translocate into the nucleus, where it binds transcription factors to induce gene expression. This includes promoting anti-amyloidogenic α-secretase expression (blue mRNA) and inhibiting pro-amyloidogenic β-secretase expression. (B) Dysfunctional Wnt, Amyloidogenic Processing, and Tau Hyperphosphorylation—The LRP6 antagonist, Dickkopf 1 (DKK1), prevents Wnt-induced GSK3β inhibition (broken red line). Thus, (i) GSK3β causes β-catenin depletion, contributing to a decrease in α-secretase expression and increase in β-secretase expression (red mRNA). In addition, (ii) GSK3β phosphorylates the intracellular domain of Amyloid Precursor Protein (APP), making APP a better substrate for β-secretase and further promoting amyloidogenic processing and the production of Amyloid β (Aβ) by β- and γ-secretase. Aβ inhibits Frizzled and induces DKK1 expression to feedback and prevent GSK3β inhibition (broken red line). Aβ also forms neurotoxic extracellular plaques. The leftover phosphorylated APP Intracellular Domain (p-AICD) induces GSK3β expression. Finally, (iii) GSK3β, also known as Tau Kinase I, phosphorylates tau, contributing to microtubule instability and to the formation of neurotoxic oligomers and phospho-tau Neurofibrillary Tangles (NFT). (C) Human Neuropathological and Genetic Data Are Consistent with the Wnt/GSK3β Model of AD—In the Alzheimer brain, as compared to the healthy aged brain, the levels and activities of Wnt-signaling components are indicative of pathway hypoactivity: DKK1 levels are elevated, LRP6 levels are reduced, GSK3β activity is high, and β-catenin is depleted. Furthermore, *LRP6* loss-of-function and *GSK3β* gain-of-function alleles are risk factors for AD.

7.3.4b Dysfunctional Wnt-signaling causes the production of Aβ

Amyloid plaques, aggregates of the amyloid β (Aβ) peptide, are the primary pathological hallmark of AD. Aβ is formed by the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase (Figure 7.10B). This amyloidogenic processing is in contrast to nonamyloidogenic processing, in which α-secretase replaces β-secretase and cleaves APP within the Aβ domain such that no Aβ is produced [319] (Figure 7.10A). By altering α- and β-secretase gene expression, as well as by decreasing APP phosphorylation, Wnt-signaling shifts APP metabolism away from amyloidogenic processing and protects against Aβ neuropathology [310, 311, 320-323].

With respect to secretase gene expression, Wnt-signaling downregulates the sole β-secretase gene, *BACE1* [319, 324], and upregulates the primary neuronal α-secretase gene, *ADAM10* [319, 325]. In APP-overexpressing mice, GSK3β inhibition has been shown to decrease *BACE1* expression and activity, thereby reducing amyloid plaque load [326]. Furthermore, in cultured neurons, activating Wnt-signaling, by using Wnt ligands or overexpressing β-catenin, is sufficient to increase *ADAM10* expression [325] and decrease *BACE1* expression, again reducing Aβ levels [324]. These data are consistent with a model in which dysfunctional Wnt-signaling in the AD brain causes GSK3β-mediated β-catenin depletion, which leads to a pathological decrease in the ratio of α-secretase to β-secretase expression (Figure 7.10Bi) and, thus, to an increase in the amyloidogenic processing of APP to Aβ [310, 311, 317, 323-328].

Wnt-signaling further suppresses amyloidogenic processing by inhibiting APP phosphorylation. Specifically, Wnt-signaling inhibits GSK3β, which otherwise phosphorylates APP on Thr668 [329, 330], contributing to the elevated p-Thr668 APP levels that are observed in the human AD brain [331] (Figure 7.10Bii). The direct consequences of Thr668 phosphorylation are two-fold. First, p-Thr668 APP is a better substrate for β-secretase than unphosphorylated APP [331]. Second, if the APP intracellular domain (AICD)—which contains Thr668 and is produced in conjunction with Aβ by γ-secretase-mediated cleavage—is phosphorylated, it can translocate into the nucleus to upregulate *GSK3* gene expression [332] (Figure 7.10B). In this way, dysfunctional Wnt-signaling permits the phosphorylation of APP by GSK3β, leading to both an increase in Aβ production and an increase in *GSK3* expression, establishing a positive feedback loop.

As predicted by this model, inhibiting Wnt-signaling with the LRP6 inhibitor, Dicckopf-1 (DKK1), increases the amyloidogenic processing of APP and impairs learning and memory in mice [324, 333-336], whereas activating Wnt-signaling with different GSK3β inhibitors decreases *BACE1* expression, decreases APP phosphorylation, decreases Aβ production, prevents neurodegeneration, and reduces learning and memory [326, 332, 337-341].

7.3.4c Aβ causes dysfunctional Wnt-signaling

Aβ, in turn, can inhibit Wnt-signaling to establish another positive feedback loop. Treatment of rat neurons *in vitro* with Aβ induces the expression of DKK1 and increases GSK3β activity, thereby decreasing β-catenin levels and contributing to the death of neurons [320, 333, 335, 336, 342]. Importantly, activation of Wnt-signaling via a variety of mechanisms—by treatment with Wnt ligands, neutralization of DKK1, or inhibition of GSK3β—appears sufficient to protect neurons against β-catenin depletion and, ultimately, death [320, 342, 343].

Not only does Aβ indirectly inhibit the initiation of Wnt-signaling by increasing the expression of the LRP6 antagonist, DKK1 [333-335, 342, 344], but it also directly blocks the binding of Wnt ligands to the other half of the LRP6-Fz receptor pair. Using cultured mouse neurons, Magdesian and colleagues demonstrated that Aβ competitively inhibits the binding of Wnt ligands to Fz and, consequently, prevents β-catenin from translocating into the nucleus to induce Wnt target gene expression [345] (Figure 7.10B). Aβ also increases GSK3β activity leading to neurodegeneration [309-311, 320, 323, 342]. Importantly, interventions that either block the interaction between Aβ and the Wnt receptors, or those that circumvent the Aβ blockade and activate Wnt-signaling downstream of LRP6- Fz, protect neurons against Aβ toxicity [309-311, 320, 323, 342, 345]. Some examples are as follows: a synthetic soluble peptide homologous to Fz competitively inhibited Aβ binding to Fz and, thereby, protected against β-catenin depletion [345]; upstream activation of Wnt-signaling using competitive amounts of exogenous Wnt ligands (Wnt3a or Wnt7a) prevented Aβ-induced neuron apoptosis; downstream activation of Wnt-signaling using multiple different GSK3β inhibitors also prevented Aβ-induced neurodegeneration [320, 343, 346].

7.3.4d An LRP6 deletion model supports the Wnt/GSK3β-Aβ feedback loop

An *LRP6* deletion mouse model provides further support for the hypothesis that dysfunctional Wnt-signaling and Aβ constitute two halves of a positive feedback loop. Liu and coworkers demonstrated that conditional deletion of *LRP6* in mouse neurons increased levels of β-secretase cleavage products and precipitated the formation of Aβ plaques, consistent with the notion that decreased Wnt-signaling promotes the formation of amyloid pathology. The neuropathological changes were associated with significant memory deficits, similar to those exhibited by more common mouse models of AD [322]. Importantly, Aβ, in turn, decreased *LRP6* expression, thus validating the positive feedback loop model in which dysfunctional Wnt-signaling causes an increase in Aβ, and vice versa.

These mouse data paralleled those from human patients with AD. Liu et al. not only found (1) lower LRP6 and β-catenin levels in the post-mortem brains of AD patients relative to age-matched control brains (Figure 7.10C), but also (2) a negative correlation between LRP6 and Aβ levels in these brains and (3) a positive correlation between LRP6 levels and Mini-Mental State Examination (MMSE) scores, a test in which higher scores indicate better cognitive function [322]. Thus, the level of Wnt-signaling dysfunction may predict the degree of neuropathology and cognitive impairment in AD patients.

7.3.4e Human neuropathological and genetic data support the Wnt/GSK3β model

Not only are LRP6 levels reduced in the post-mortem brains of AD patients, but DKK1 levels are also elevated [314, 323, 342]. The simultaneous decrease in the Wnt receptor (LRP6) and increase in its inhibitor (DKK1) cooperatively downregulates Wnt-signaling and increases GSK3β activity in patients' brains [309, 311, 314, 347, 348]. The genetic data concur. Specifically, a loss-of-function mutation in *LRP6* has been identified as a risk factor for AD [349], as have gain-of-function mutations in the *GSK3* gene [350] (Figure 7.10C).

Furthermore, evidence suggests that the strongest known genetic risk factor for AD in humans, the *ApoE4* allele [351], may negatively impact Wnt-signaling. Similar to Aβ, the ApoE4 protein increases DKK1 expression, binds to the LRP6-Fz receptor complex, activates GSK3β, and promotes the amyloidogenic processing of APP [310, 323, 327, 352-355]. Therefore, there is a case to be made that ApoE4 either sparks the positive feedback loop between Wnt-signaling and Aβ, decreases the threshold for the establishment of the feedback loop, and/or accelerates the rate at which the loop spirals into life-altering disease.

7.3.4f GSK3β links Aβ to p-tau

In addition to contributing to the build-up of amyloid plaques, the first of the two pathological hallmarks of AD, dysfunctional Wnt-signaling may also contribute to the development of the second hallmark of AD, phospho-tau (p-tau) Neurofibrillary Tangles (NFTs). GSK3β, alternatively known as Tau Kinase I, is thought to be the mechanistic link between Aβ and p-tau [310, 311, 329, 347, 356]. By inhibiting Wnt-signaling, Aβ increases GSK3β activity [309-311,

320, 323, 342]. In turn, GSK3β phosphorylates tau on a set of residues known to be phosphorylated in AD [310, 311, 329, 347, 356]. This results in two events. First, tau dissociates from microtubules, disabling tau's physiological function as a microtubule-associated protein and thereby contributing to cytoskeleton instability [as an aside, it's worth noting that recent data suggest tau functions as more than just a microtubule-associated protein and that tau loss-of-function can contribute to a broader array of cellular defects than previously thought, including brain insulin resistance [357]. Second, hyperphosphorylated tau aggregates into neurotoxic oligomers that exert further harmful effects on the cell, such as inducing mitochondrial dysfunction, oxidative stress, neuroinflammation, and apoptosis [358-360] (Figure 7.10Biii).

Experiments conducted in two different animal models of AD, GSK3β mice and APP mice, build a strong case for the serial connection amongst Aβ, GSK3β, and p-tau. First, conditional overexpression of *GSK3* in the cortices and hippocampi of adult mice has been shown to reduce levels of nuclear β-catenin and increase levels of p-tau [356]. The GSK3β-induced increase in p-tau pathology is further associated with an increase in neuronal apoptosis and performance deficits in the Morris water maze test of spatial memory [356, 361]. Second, mice overexpressing APP have increased Aβand p-tau loads, along with memory deficits. However, inhibition of GSK3β in these APP mice is sufficient to protect against p-tau pathology and against cognitive impairment [338]. The neuroprotective and anti-p-tau effects of GSK3β inhibition in the APP mouse model have been replicated by multiple independent groups [339, 341]. In short, the two murine models suggest that GSK3β/Tau Kinase I, a central player in Wnt-signaling, links the Aβ and p-tau pathologies of AD.

7.3.5 α-synuclein

7.3.5a Human neuropathological and genetic data suggest overlapping pathology between Alzheimer's and Parkinson's disease

Neither AD nor PD are monolithic disease entities; it is likely that each is composed of several subtypes that have yet to be effectively characterized. At least some of the putative AD subtypes overlap in pathology with those of PD, and vice versa. More than half of patients with AD present with Lewy bodies, aggregates of α-synuclein that are the PD equivalent of Aβ plaques [308, 312]. Furthermore, α-synuclein is a component of AD plaques themselves. In fact, the creatively named non-Aβ component (NAC) of plaques is a fragment of α-synuclein [362, 363]. Thus, α-synuclein lesions are present in the AD brain as distinct Lewy body structures and as part of amyloid plaques.

Complementarily, classic AD inclusions are observed in the PD brain. Specifically, in PD patients, p-tau tends to aggregate in the substantia nigra and other PD-associated brain regions [304, 308, 312, 364]. This presence of p-tau tangles also correlates with increased GSK3β activity, an observation that suggests GSK3β may be responsible for tau phosphorylation in PD, as it is in AD [303, 304, 328, 348, 365]. An extension of this logic is that dysfunctional Wnt-signaling may be a convergence point for the world's two most common neurodegenerative disorders.

The genetic evidence also suggests that GSK3β, tau, and α-synuclein can synergistically interact in neurodegeneration. As in AD, polymorphisms in the genes that code for GSK3β and tau (*MAPT*) are risk factors for PD [312, 328, 350, 366, 367]. Furthermore, there is a genetic interaction between *MAPT* and the α-synuclein gene (*SNCA*) in which the high-expression *MAPT* haplotype (H1) and a polymorphism in *SNCA* synergistically increase PD risk [367]. Notably, in this study,

only PD patients with the H1/H1 *MAPT* haplotype went on to develop PD with dementia, hinting that this may be an instance in which the pathology and symptoms of a PD subtype overlap with those more typical of AD [367].

More relevant to this review article, the *SNCA* gene also affects AD risk. Some *SNCA* polymorphisms double the risk of AD [368, 369], whereas others decrease the risk of AD [370]. With respect to the latter, a retrospective study conducted by Xia and colleagues showed that a particular allele in the *SNCA* promoter was enriched 4-fold in cognitively healthy *ApoE4* carriers as compared to *ApoE4* carriers with AD, suggesting that this *SNCA* polymorphism has a protective effect against the strongest known risk factor for AD. This interaction was dose-dependent as the presence of the *SNCA* allele decreased AD risk by 3-fold in *ApoE4* heterozygotes and by 10-fold in *ApoE4* homozygotes [370]. The fact that *SNCA* mutations affect AD risk is consistent with the hypothesis that α-synuclein plays a role in the development of AD, at least in some instances.

7.3.5b α-Synuclein induces amyloid pathology, possibly in a Wnt/GSK3β-dependent manner, and is in positive feedback with Aβ

Studies using cultured neurons have demonstrated that either exogenous treatment with α-synuclein or α-synuclein overexpression is sufficient to increase the production and secretion of Aβ [307, 371]. One mechanism by which α-synuclein could increase Aβ levels is by activating GSK3β, as suggested by mouse experiments that show that α-synuclein overexpression increases GSK3β activity [303, 328]. Exactly how α-synuclein activates GSK3β is a matter that requires further investigation; however, several lines of *in vitro* and mouse data imply that α-synuclein in

neurons could induce GSK3β-activating ROS [372-375] (Figure 7.11Ai) and decrease the production neuroprotective canonical Wnt ligands by astrocytes [376-381] (Figure 7.11Aii; for an excellent review of the role of Wnt-signaling in neuron-microglia-astrocyte crosstalk in neurodegeneration, see [382]). Although the dominant mechanism by which α-synuclein induces GSK3β *in vivo* is unclear, the observation that intracranial injections of α-synuclein increase β-secretase and Aβ levels in mice [307] is, at minimum, consistent with the model presented in Figure 7.10B and with the hypothesis that α-synuclein-induced Aβ production is mediated by the Wnt/GSK3β axis.

In turn, exogenous treatment with Aβ, even at concentrations as low as 1 μM, has been shown to increase α-synuclein levels in neurons [371]. Although the mechanisms by which Aβ reciprocally induces α-synuclein likewise remains a gap in the literature, it is worth noting that upregulation of Wnt-signaling via β-catenin overexpression or GSK3β inhibition protects PD models from developing α-synuclein pathology and motor deficits [383, 384]. Furthermore, *in vitro*, *Drosophila*, mouse, and human data collectively suggest that GSK3β specifically phosphorylates Ser129 of α-synuclein (Figure 7.11A1), a post-translational modification predominant in Lewy bodies and in the PD brain that may enhance α-synuclein aggregation and/or neurotoxicity [385-388]. GSK3β is also a known inhibitor of autophagy [321, 389, 390], a ubiquitous cellular recycling process required for the effective clearance of excess α-synuclein [391, 392] (Figure 7.11A2). Therefore, it is plausible that Aβ-induced GSK3β activation (Figure 7.10B) completes an α-synuclein-Aβ feedback loop relevant in some cases of AD.

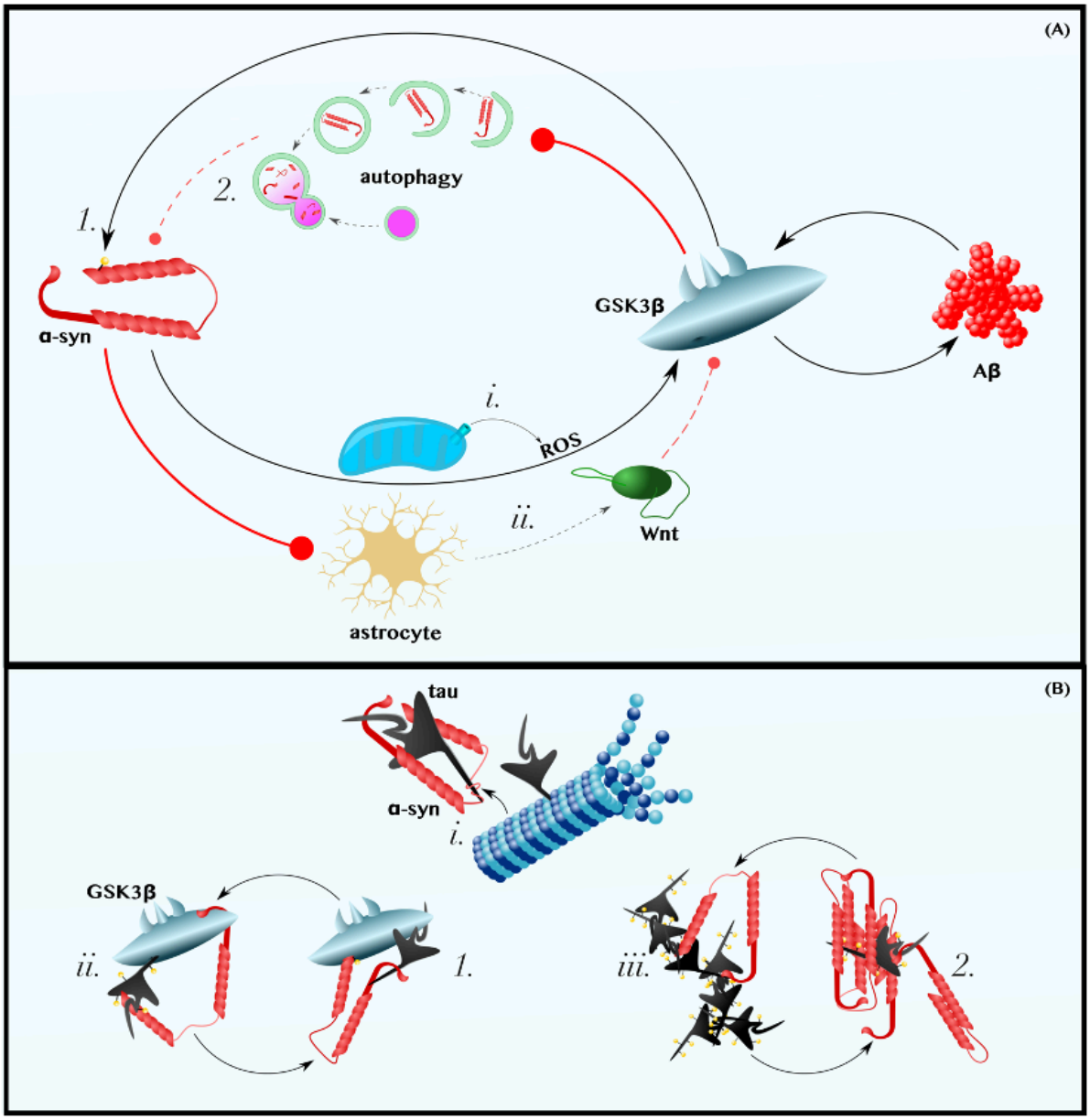

(A)
autophagy
1.
2.
α-syn
GSK3β
Aβ
i.
ROS
ii.
Wnt
astrocyte
(B)
tau
α-syn
i.
GSK3β
ii.
1.
iii.
2.

Figure 7.11 α-Synuclein is in positive feedback with the Aβ and tau

(A) α-Synuclein is in Positive Feedback with Aβ—α-synuclein may induce oxidative stress and promote astrocytic dysfunction. Thus, perhaps by (i) increasing the levels of cytoplasmic ROS and (ii) decreasing those of extracellular astrocyte-derived Wnt ligands, α-synuclein activates GSK3β and induces the production of Aβ. (For a more comprehensive discussion about the role of Wnt-signaling in neuron-glia crosstalk in neurodegeneration, see L'Episcopo et al. 2018.) In turn, Aβ activates GSK3β, which (1) phosphorylates α-synuclein on Ser129 and (2) may impair the autophagic clearance of α-synuclein. (B) α-Synuclein is in Positive Feedback with Tau—α-synuclein can (i) bind tau's microtubule binding domain, causing tau to disassociate from microtubules, (ii) recruit GSK3β to tau and, thereby, promote tau hyperphosphorylation, and (iii) directly seed or chaperone the pathological aggregation of p-tau. Tau can reciprocate by (1) recruiting GSK3β to α-synuclein, thereby permitting pathogenic Ser129 phosphorylation, and by (2) promoting the aggregation of α-synuclein. Dashed and solid lines indicate regulatory mechanisms that are, respectively, impaired and enhanced in Alzheimer disease.

7.3.5c α-synuclein, directly and via GSK3β, induces tauopathy and is in positive feedback with p-tau

α-synuclein and tau interact directly [308, 393]. Specifically, α-synuclein binds tau within tau's microtubule-binding domain [393]. Even were this interaction not sufficient to cause tau to disassociate from microtubules, the binding of α-synuclein to tau induces the phosphorylation of tau on Ser262, a post-translational modification observed in the AD brain that causes tau to release from microtubules, contributing to cytoskeleton instability [393]. Subsequently, α-synuclein can serve as a necessary cofactor to help p-tau form oligomers and, eventually, tangles [301, 394]. Thus, as reviewed by Moussaud and colleagues, there are at least three ways by which α-synuclein can instigate and aggravate tauopathy: by blocking the interaction between tau and microtubules, thereby interfering with tau's physiological function (Figure 7.11Bi), by recruiting kinases that promote tau hyperphosphorylation (Figure 7.11Bii), and by seeding or chaperoning the aggregation of tau into neurotoxic oligomers and fibrils [312] (Figure 7.11Biii).

With regard to the kinase mechanism listed above, GSK3β/Tau Kinase I may play a particularly important role in the relationship between α-synuclein and tau. Not only does α-synuclein interact with tau, but both proteins also interact with, and are phosphorylated by, GSK3β [303, 388]. Thus, α-synuclein can recruit GSK3β to tau, leading to tau hyperphosphorylation (Figure 7.11Bii). As this model predicts, exogenous treatment of cultured cells with α-synuclein increased levels of p-tau, this phenomenon being blocked by the inhibition of GSK3β [306]. Similar findings have been produced in mice in which the overexpression of α-synuclein is sufficient to induce GSK3β-mediated p-tau pathology [303]. Reflecting on the stimulatory effect of α-synuclein on GSK3β, as well as that of Aβ on GSK3β (Figure 7.11B), we can elaborate upon our model: GSK3β can be

conceptualized as the convergence point of a Y-shaped cascade in which either Aβ or α-synuclein can activate and/or recruit GSK3β to induce tau pathology.

Similar to the mutualistic case of Aβ and α-synuclein, p-tau can promote α-synuclein pathology [301, 308, 395]. Using multiple different cell models, [395] demonstrated that tau enhanced the aggregation of α-synuclein. In these experiments, tau overexpression also reduced cell viability in an α-synuclein-dependent manner [395], perhaps by promoting the GSK3β-mediated neurotoxic phosphorylation of α-synuclein on Ser129 [385-388], and promoted the secretion of α-synuclein [395]. Thus, tau can complete an intracellular positive feedback loop with α-synuclein, possibly by facilitating the pathogenic phosphorylation of α-synuclein Ser129 by GSK3β (Figure 7.11B1) and/or by promoting α-synuclein's aggregation (Figure 7.11B2), and tau might also support the prionic cell- to-cell propagation of α-synuclein (not shown in Figure 7.11). Independent of the exact mechanisms, the relevance of tau on α-synuclein pathology and its attending symptoms has been demonstrated *in vivo*. In mice, the transgenic expression of tau enhances the formation of α-synuclein inclusions and the corresponding Parkinsonian phenotype [301].

7.3.6 Type-III diabetes

7.3.6a Overview of insulin signaling and its role in the brain

Several lines of evidence suggest that, in the central nervous system, insulin does much more than promote glucose uptake. Insulin is a neuromodulator, affecting the reuptake and production of particular neurotransmitters [396, 397]; insulin regulates food intake and reproduction by acting on the hypothalamus to alter endocrine system function [397]; and, glucose transport into neurons is largely insulin-independent. Building upon this last key piece of evidence, neuron energy utilization also correlates poorly with the heterogeneous distribution of Insulin Receptor (IRs) throughout the brain, further suggesting that insulin's primary functions in the brain include more than glucose uptake [396]. And, although IRs are also concentrated in the hypothalamus, olfactory bulb, and cerebellum, it's notable that IRs are particularly densely packed in the hippocampus and cerebral cortex, two brain regions important in learning and memory that are critically impacted by AD [396-398].

The insulin signaling cascade is initiated when insulin binds to the IR, a heterotetrameric receptor tyrosine kinase that autophosphorylates in order to recruit the adaptor protein IR Substrate (IRS). IRS subsequently recruits and activates Phosphoinositide 3-Kinase (PI3K), a lipid kinase that generates the second messenger Phosphatidylinositol (3,4,5)- trisphosphate (PIP3). PIP3 can diffuse along the membrane to activate Phosphoinositide-Dependent Kinase 1 (PDK1), which phosphorylates and activates the terminal kinase in the core of this cascade, AKT [399] (Figure 7.12A).

AKT regulates an expansive set of pathways and processes, only some of which will be discussed in the following section. AKT (i) regulates translocation of GLUT3, the canonical neuronal glucose transporter, and of GLUT4, which is also essential in neurons [400], to the plasma membrane [401, 402]. At the axon terminal and post-synaptic density, the insulin-AKT pathway (ii) modulates catecholamine release and uptake, the trafficking of ion-gated channels, and the expression and localization of neurotransmitter receptors [255, 403]. Finally, AKT (iii) is a potent GSK3β inhibitor [404] (Figure 7.12B). Each of these mechanisms will be discussed further in the following subsections.

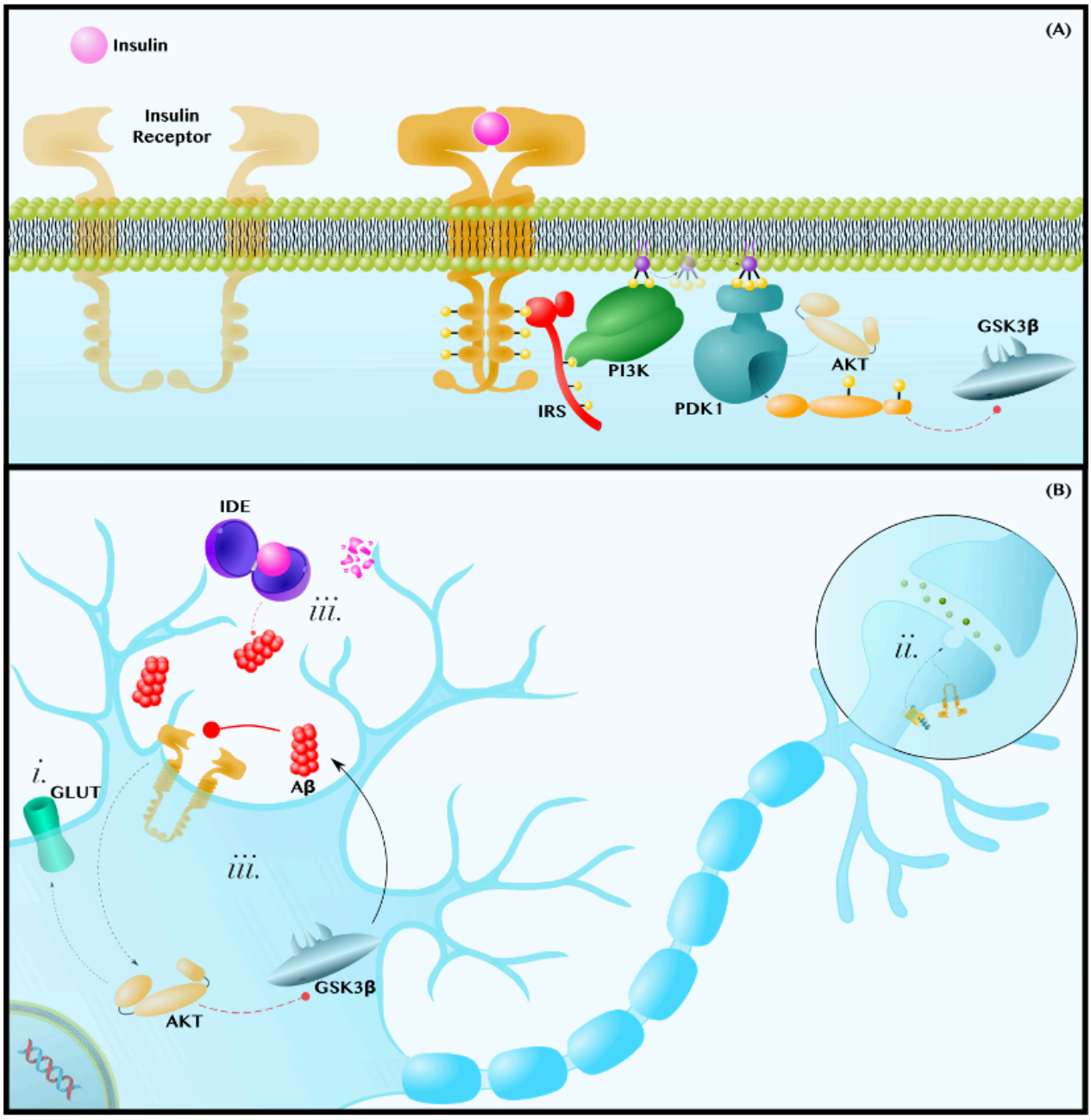

(A)
Insulin
Insulin Receptor
IRS
PI3K
PDK1
AKT
GSK3β
(B)
IDE
i.
GLUT
ii.
iii.
iii.
Aβ
AKT
GSK3β

Figure 7.12 Insulin resistance exacerbates the pathology of Alzheimer disease

(A) Insulin-AKT Pathway—Insulin binds to the Insulin Receptor tyrosine kinase, which autophosphorylates and binds the adaptor protein, Insulin Receptor Substrate (IRS). IRS recruits Phosphoinositide 3-Kinase (PI3K), which phosphorylates PIP_2 into PIP_3. PIP_3 diffuses along the membrane to activate Phosphoinositide-Dependent Kinase 1 (PDK1), which activates AKT. AKT phosphorylates many enzymes; this includes inhibiting GSK3β. (B) Insulin Resistance Contributes to Neuropathology—Insulin resistances (i) causes a decrease in AKT-mediated translocation of GLUT transporters to the membrane. This contributes to the decreased glucose metabolic rate and mitochondrial dysfunctions observed in Alzheimer and Parkinson's brains. Insulin-AKT signaling is critical in synaptic transmission, as is Wnt-signaling. Therefore, insulin resistance (ii) may synergize with dysfunctions in Wnt-signaling to decrease synaptic transmission and synapse integrity. Lastly, insulin resistance (iii) can contribute to hyperinsulinemia and the competitive inhibition of Insulin Degrading Enzyme (IDE), which also degrades Aβ. Since Aβ inhibits insulin-AKT signaling, it can establish a positive feedback loop in which Aβ inhibits insulin signaling to decrease AKT activity, increase GSK3β activity and, thus, further increase Aβ levels. Dashed and solid lines indicate regulatory mechanisms that are, respectively, impaired and enhanced in Alzheimer disease.

7.3.6b Lack of energetic substrates as an exacerbating factor for AD

Even preclinically, patients with AD show widespread impairment in glucose metabolic rates [251], a deficiency associated with decreased levels of GLUT1 and GLUT3 [405], which import glucose across the blood-brain barrier and into neurons, respectively. As the brain can only use either glucose or ketones, and ketones are not normally available as a fuel, insulin resistance and the ensuing decrease in GLUT membrane expression (Figure 7.12Bi) can decrease mitochondrial ATP production and all ATP-dependent maintenance processes that are critical to neuron survival [406, 407].

Animal models support the relevance of GLUT transporter underexpression in AD, as well as the potential involvement of dysfunctional Wnt-signaling in this process. For example, overexpression of GLUT3, which is regulated, in part, by AKT [402], helps rescue *Drosophila* from the morphological and behavioral features associated with Aβ toxicity [260]. Furthermore, in a mouse model of AD, Nishida and colleagues demonstrated that decreased GLUT1 expression at the blood-brain barrier was associated with decreased cerebral blood flow, increased Aβ accumulation, and memory impairment [261]. Interestingly, Wnt-signaling has been identified as necessary for GLUT1 expression at the blood-brain barrier [408], and Pan and colleagues showed that inhibition of GSK3β in AD mice has precisely the opposite effects to those just described in that GSK3β inhibition increased cerebral blood flow, prevented Aβ accumulation, and rescued memory impairment [341]. The complementary findings of the two mouse studies, in combination with the fact that Wnt ligands have been observed to increase AKT activity and neuronal glycolytic rate [409], hints at the possibility that dysfunctions in the insulin-AKT and Wnt-signaling pathways may cooperate to contribute to glucose metabolism deficiency in AD.

7.3.6c Insulin resistance and Wnt-signaling in synaptic dysfunction

As insulin regulates the release and reception of neurotransmitters, cerebral insulin resistance can contribute to a decrease in synaptic activity and density [255, 403, 410, 411] (Figure 7.12Bii). In *Xenopus* tadpoles, the expression of a dominant- negative IR decreased excitatory post-synaptic potentials and synaptic density [255]. Conversely, activation of the insulin-AKT axis, by pharmacologically stimulating AKT or PI3K, increased synaptic density and rescued aberrant synaptic plasticity in wildtype and AD rodents [264, 412].

At the synapse, the effects of dysfunctional Wnt-signaling have been shown to be analogous to those of dysfunctional insulin-signaling. Specifically, blocking the initiation of Wnt-signaling with DKK1 induced synaptic loss in mice [334, 344]. Furthermore, as with AKT activation [264], direct pharmacological activation of Wnt-signaling was sufficient to rescue aberrant synaptic plasticity [334, 344]. This, along with the suggestion of crosstalk between the Wnt and AKT pathways [409, 413], raises the possibility that insulin resistance and dysfunctional Wnt-signaling may interact to induce synaptic dysfunction in cognitive decline.

7.3.6d Insulin resistance and Aβ can establish a Wnt/GSK3β-dependent positive feedback loop

Insulin Degrading Enzyme (IDE) is a cytoplasmic and secreted enzyme that degrades both insulin and Aβ in the human brain [268, 414]. Accordingly, hyperinsulinemia, which is associated with an approximately two-fold increase in AD risk [415], can competitively inhibit IDE-mediated Aβ degradation [266, 268, 414, 416]. In turn, Aβ can exacerbate hyperinsulinemia by inhibiting IDE and competing for IR binding [267, 268, 417].

But, even in those cases in which cerebral hyperinsulinemia does not initiate the accumulation of Aβ, a vicious cycle between Aβ and insulin-AKT signaling can arise once some degree of amyloid pathology has been established (Figure 7.12Biii). The De Felice group has shown that intracerebroventricular infusion of Aβ oligomers in monkeys disrupts insulin-AKT signaling in the hippocampus in a TNFα-dependent manner, leading to memory impairment [418]. In this way, Aβ releases GSK3β from AKT-mediated inhibition and, reciprocally, GSK3β increases Aβ production via the mechanisms displayed in Figure 7.10B.

It is also notable that the De Felice group later showed that intracerebroventricular infusion of Aβ oligomers caused hypothalamic dysfunction and peripheral insulin resistance in mice, again in a TNFα-dependent manner. This latter finding, in conjunction with epidemiological data showing AD increases an individual's risk of developing T2DM, suggests yet another pathological feedback loop in which systemic insulin resistance increases Aβ production, leading to Aβ-mediated hypothalamic inflammation that further exacerbates systemic insulin resistance [419].

7.3.6e The AKT paradox

Obviously, Figure 7.12 is a simplification of insulin resistance pathology in the AD brain. What is not as obvious is how it is a simplification. Not only are pathways and relationships among proteins necessarily omitted, but there is also a lack of consensus on the fundamental nature of key relationships. An important and illustrative example is that AKT may be either underactive or overactive in the post-mortem human AD brain [271, 420].

While this AKT paradox remains to be resolved, one hypothesis is that the opposite dysfunctions in AKT activity are time-dependent. For example, intracellular and extracellular Aβ may have different effects on AKT activity, with intracellular Aβ (not explicitly shown in Figure 7.12B) accumulating well before extracellular Aβ [270]. Intracellular Aβ can interfere with the interaction between PDK1 and AKT, contributing to a decrease in AKT activity and to disease progression [270, 271]. However, as extracellular Aβ builds up later, a tipping point [possibly one that is neuron-specific and heterogenous across the brain [420] may be reached whereby Aβ binds to IRs and constitutively overstimulates AKT [417, 421, 422]. Rather than being neuroprotective, this 180-degree flip may be pathogenic in other ways, including saturating pathway activity, such that the pathway is no longer responsive to insulin, and inducing mTOR1-mediated IRS inhibition, thus reinforcing insulin resistance [417, 423]. Moreover, Aβ binding to IRs causes a dramatic migration of IRs away from neurites to the soma [417], impairing synaptic integrity and compounding spatial complexity on top of temporal complexity.

Evidently, the AKT paradox adds a major qualification to the model presented in Figure 7.12B, which we presented as is for the following two reasons: (1) decreased GLUT transporter expression, decreased synaptic integrity, and increased GSK3β activity have been more consistently observed in the AD brain [311, 323, 347, 405] and (2) pharmacological activators of AKT have demonstrated therapeutic efficacy in *Drosophila* and mouse models of AD [264, 424], whereas the same cannot be said for AKT inhibitors. It is important to acknowledge the AKT paradox as a representative example of the nuance present within even a single model of AD. Appreciating this nuance will help us better appreciate the true complexity of AD that arises out of an interrelationship among the models.

7.3.7 An integrated perspective and concluding remarks

In this review article, we began by summarizing the cellular, animal, and human work that demonstrate dysfunctional Wnt-signaling can contribute to the development of AD and its two pathological hallmarks, Aβ plaques and p-tau tangles. We next described how the canonical PD-associated protein α-synuclein may be locked in pathological positive feedback loops with Aβ and tau. Finally, we discussed some of the mechanisms by which insulin resistance in the brain, ‘‘type 3 diabetes,’’ may contribute to development and exacerbation of AD. Throughout each section, we attempted to highlight some of the ways in which each model interacts with the others. These interrelationships, summarized in Figure 7.13, make it clear that the pathology of AD is not a linear cascade, nor a simple feedback loop, but rather a network of cross-talking models and overlapping vicious cycles.

Given the cooperative and reinforced nature of this complex network, it is no surprise that the prototypical monotherapeutic approach to AD has reliably failed. Certainly, drugs that target key nodes within the network, such as GSK3β inhibitors [321, 425, 426] or AKT activators [264, 424], have shown promise in animal models, and this important work affords us valuable mechanistic insights. However, these pre-clinical successes generally have not translated into clinical success, at least not with the same degree of efficacy. This is likely because animal models harboring distinct AD-causing mutations and dysfunctions in particular linear pathways do not accurately recapitulate the complex pathologies underlying sporadic human AD. In brief, we are proposing that the single-target silver-bullet approach to AD drug discovery is doomed to fail and that we may only be able to treat or prevent AD by developing new multifaceted treatment options.

Further complicating matters, the initial movers of sporadic human AD are likely highly individual. As examples, only about half of AD patients present with Lewy Body/α-synuclein pathology [308] and there is evidence to suggest that diabetes may specifically predispose carriers of the *ApoE4* risk allele to develop AD [269, 427]. If AD is, indeed, composed of many different subtypes, then even imagining AD as a network of reinforcing positive feedback loops, as we have done here, underestimates the pathology. We may not only need multifaceted treatment options, but personalized ones.

The cost of continuing to simplify AD pathology is a continuation in the rapidly rising prevalence of AD. It is, therefore, critical that the global biomedical community take steps towards thinking more comprehensively about the mechanisms underlying AD, for only by doing so can we hope to develop multifaceted, and perhaps one day individualized, therapies to prevent or treat this devastating disease and reverse the worldwide neurodegeneration epidemic.

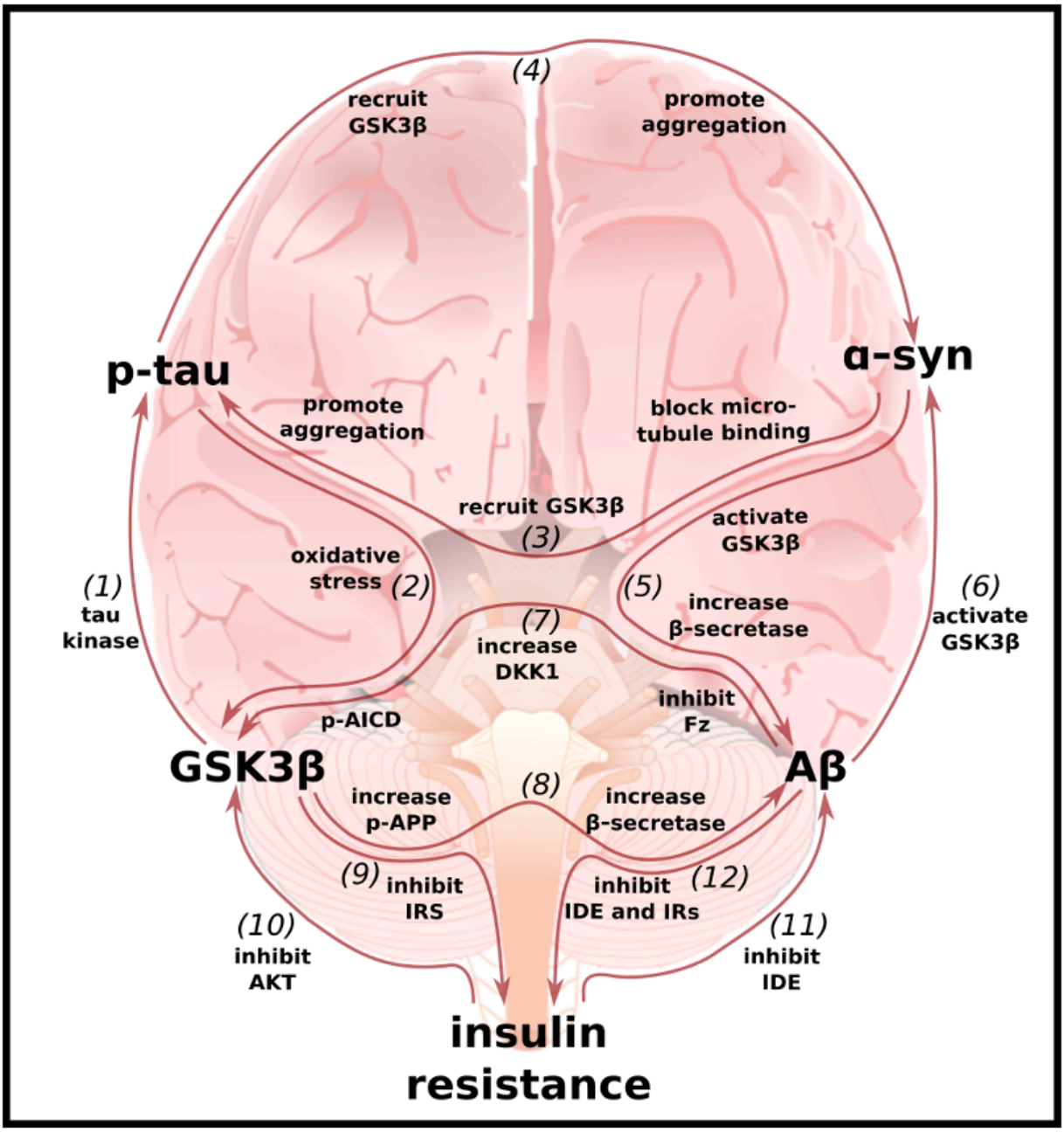

(4)
recruit GSK3β
promote aggregation
p-tau
α-syn
promote aggregation
block micro-tubule binding
recruit GSK3β
(3)
activate GSK3β
oxidative stress (2)
(1) tau kinase
(5)
increase β-secretase
(6) activate GSK3β
(7) increase DKK1
inhibit Fz
p-AICD
GSK3β
Aβ
(8)
increase p-APP
increase β-secretase
(9) inhibit IRS
inhibit IDE and IRs (12)
(10) inhibit AKT
(11) inhibit IDE
insulin resistance

Figure 7.13 Multi-loop model of Alzheimer disease: An integrated perspective on the Wnt/GSK3β, α-synuclein, and type 3 diabetes hypotheses

(1) GSK3β, also known as Tau Kinase 1, phosphorylates tau. (2) In turn, p-tau may increase GSK3β activity by inducing oxidative stress.. (3) α-synuclein can also contribute to tau pathology by binding to tau's microtubule binding domain, recruiting GSK3β to tau, and helping to promote pathological p-tau aggregation. (4) In reciprocation, tau can promote α-synuclein's phosphorylation by GSK3β and α-synuclein aggregation. (5) In addition to facilitating tauopathy, α-synuclein can promote Aβ production by increasing GSK3β activity and β-secretase levels. (6) In turn, Aβ can increase α-synuclein levels, possibly by stimulating GSK3β. (7) Aβ can simulate GSK3β activity by inducing the expression of DKK1 and by binding to and inhibiting Frizzled. p-AICD, a by-product of Aβ production, can increase *GSK3β* gene expression. (8) GSK3β phosphorylates APP to enable p-AICD production and to make APP a better substrate for β-secretase. GSK3β overactivity, and Wnt-signaling underactivity, also increase β-secretase levels, further promoting the generation of Aβ. (9) GSK3β can contribute to insulin resistance by phosphorylating and inhibiting IRS1. (10) In turn, insulin-AKT pathway dysfunction can contribute to an increase in GSK3β activity. (11) Because insulin and Aβ are both IDE substrates, hyperinsulinemia prevents Aβ degradation. (12) Aβ can then further exacerbate insulin resistance by preventing insulin degradation and by binding to IRs. The above figure shows only mechanisms whereby these models feedback on one another and not those additional mechanisms whereby they cooperate to intensify AD pathology, such as may be the case for glucose transporter expression and synaptic activity.

7.4 **Norwitz NG**, Querfurth H. mTOR mysteries: nuances and questions about the mechanistic target of rapamycin in neurodegenerative disease. *Frontiers in Neuroscience*. 2020

7.4.1 Statement of relevancy

The mechanistic target of rapamycin (mTOR) is popularly perceived as "bad" for brain health and brain aging because of its role in inhibiting autophagy. However, such a simplistic point of view fails to recognize the nuances scattered throughout the mTOR literature. This perspectives piece discusses both the pros and cons of mTOR activity and how they relate to developing strategic metabolic treatments for neurodegenerative disorders. As with section 7.3, this work is intended to add nuance to the field of neurodegeneration.

7.4.2 Abstract

The mechanistic target of rapamycin protein complex, mTORC1, has received attention in recent years for its role in aging and neurodegenerative diseases, such as Alzheimer's disease. Numerous excellent reviews have been written on the pathways and drug targeting of this keystone regulator of metabolism. However, none have specifically highlighted several important nuances of mTOR regulation as relates to neurodegeneration. Herein, we focus on six such nuances/open questions: (1) "Antagonistic pleiotropy" – Should we weigh the beneficial anabolic functions of mTORC1 against its harmful inhibition of autophagy? (2) "Early/late-stage specificity" – Does the relative importance of these neuroprotective/neurotoxic actions change as a disease progresses? (3) "Regional specificity" – Does mTOR signaling respond differently to the same interventions in different brain regions? (4) "Disease specificity" – Could the same intervention to inhibit mTORC1 help in one disease and cause harm in another disease? (5) "Personalized therapy" – Might genetically-informed personalized therapies that inhibit particular nodes in the mTORC1 regulatory network be more effective than generalized therapies? (6) "Lifestyle interventions" – Could specific diets, micronutrients, or exercise alter mTORC1 signaling to prevent or improve the progression neurodegenerative diseases? This manuscript is devoted to discussing recent research findings that offer insights into these gaps in the literature, with the aim of inspiring further inquiry.

7.4.3 Introduction

Neurodegenerative diseases are an accelerating pandemic. The burden of Alzheimer disease (AD) alone is staggering and climbing at a precipitous rate. 5.8 million Americans over the age of 65 suffer from AD, a number that is expected to triple to 13.8 million by 2050 [428]. AD is not alone in its ascent. Parkinson's disease (PD), the second most common form of neurodegeneration, is increasing in prevalence at a similarly alarming rate [300]. As there are currently no effective long-term treatments for these diseases, new therapies are desperately needed. One potential molecular target of such therapies is the mechanistic target of rapamycin complex 1 (mTORC1), a nutrient sensor and metabolic regulator heavily implicated in the process of aging [429-431].

While this manuscript will be primarily devoted to discussing and gaps in the literature surrounding mTORC1, a succinct overview of mTOR signaling and regulation is warranted as a preface to this discussion and is depicted in Figure 7.14. (For a more comprehensive overview, Heras-Sandoval et al. recently published an excellent review on mTOR signaling, regulation, and drug-targeting [430].) mTORC1 is composed of the proteins mTOR kinase and its regulator protein, Raptor, as well as mLST8, PRAS40, and Deptor. Its primary function is to sense intracellular nutrient status and extracellular trophic factors (including but not exclusive to insulin, shown in Figure 7.14 as an example), integrate these signals, and ultimately regulate the balance between cells' anabolic and catabolic processes. Specifically, mTORC1 is a positive regulator of protein synthesis and negative regulator of autophagy.

mTORC1 itself is regulated positively by insulin-signaling and negatively by AMPK. Insulin/Akt signaling inhibits the protein complex, TSC1/2, which itself prevents the conversion of the mTORC1 activator, Rheb, into its active GTP-bound form [432, 433]. Insulin/Akt signaling turns off TSC1/2, thereby activating Rheb and mTORC1. By contrast, AMP-activated protein kinase (AMPK) activates TSC1/2 [434] and directly inhibits mTORC1 by phosphorylating Raptor [435]. In brief, the respective growth and preservation functions of insulin and AMPK align with their respective stimulatory and inhibitory regulations of mTORC1.

mTORC1 promotes protein synthesis by phosphorylating and activating the downstream targets, 4E-BP1 and p70S6K1, which directly promote the initiation and elongation phases of translation [436]. Critically, mTORC1-mediated anabolic signaling promotes the development of neuronal synapses [437] (in part, by responding to established neuronal growth factors like BDNF [438]) and inhibits apoptosis [439, 440]. Through these two mechanisms, mTORC1 activity has the potential to promote learning and memory and protect against neurodegeneration. Correspondingly, excessive inhibition of mTORC1 can impair learning and memory and permit neuronal death [436, 441-444].

Despite these potentially positive functions of mTORC1 signaling in the brain, far more attention has been paid to its negative regulation of autophagy, an intracellular recycling process essential to maintaining neuronal integrity and protecting against neurodegenerative diseases [430, 445, 446]. mTORC1 inhibits autophagy at multiple levels, including the inhibitory phosphorylation of ULK1 and transcription factor EB (TFEB), which respectively initiate autophagy and promote the lysosomal biogenesis required to break down the contents of autophagosomes [447, 448].

Importantly, multiple independent human post-mortem studies confirm levels of phosphorylated mTOR and its downstream targets are elevated in the AD brain as compared to those of controls [449-452]. Dysregulated autophagy is also a hallmark of multiple neurodegenerative conditions [453], which is not surprising because autophagy is required to prevent the accumulation of toxic intracellular protein aggregates that contribute to neurodegenerative diseases, such as amyloid-β (Aβ) [454-456], phospho-tau [457-459], α-synuclein [218, 460, 461], and mutant huntingtin [462]. Autophagy is also required to recycle mitochondria and prevent mitochondrial dysfunction [463, 464], another hallmark of neurodegenerative diseases, and one which can further lead to the pathologies of oxidative stress and inflammation [30, 273, 465]. Given these data and the clinical burden of neurodegenerative disease, it's reasonable that translational research generally focuses on the inhibition of mTOR (and promotion of autophagy), rather than its activation.

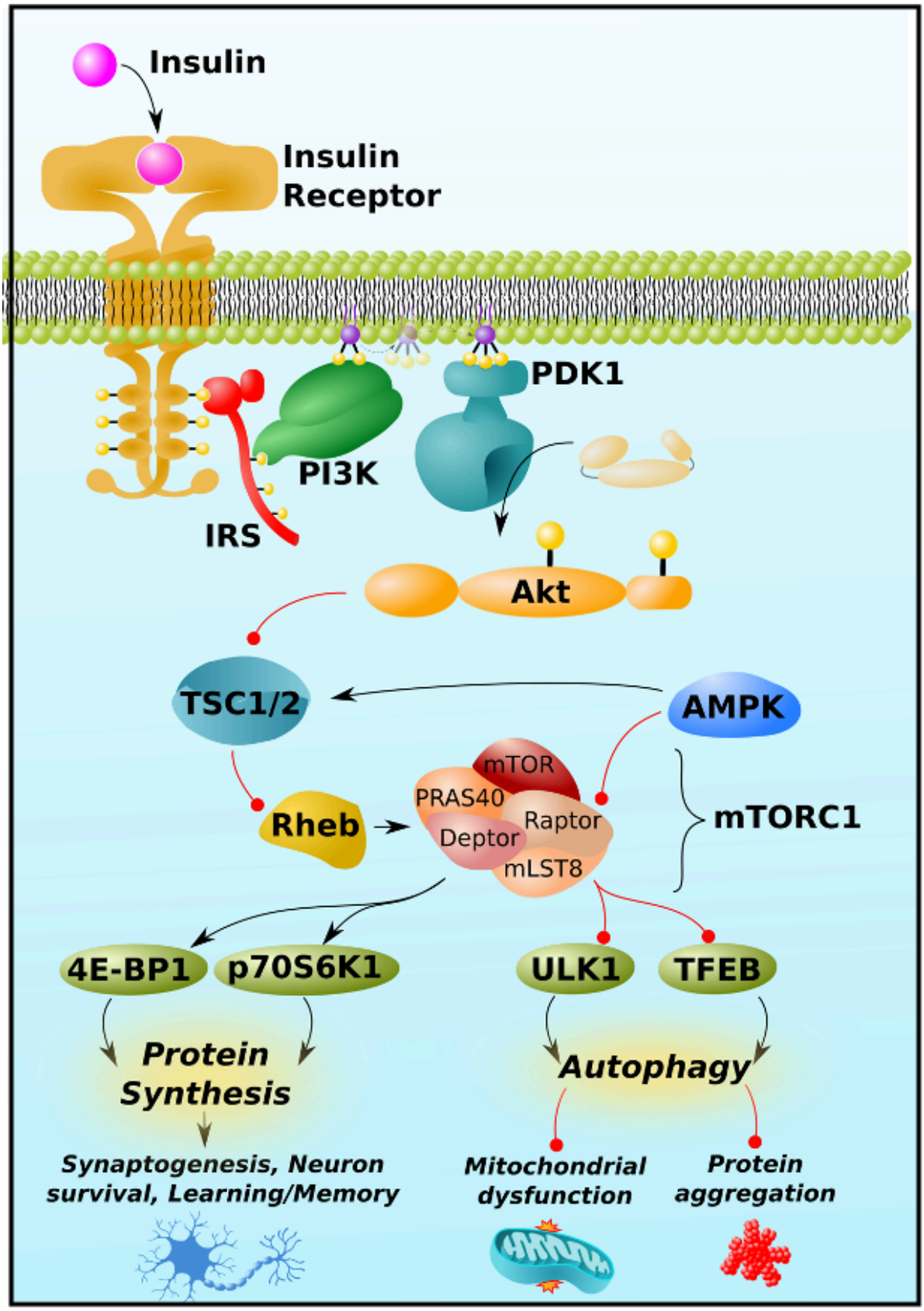

Insulin
Insulin Receptor
PDK1
PI3K
IRS
Akt
TSC1/2
AMPK
mTOR
PRAS40
Raptor
Deptor
mLST8
mTORC1
Rheb
4E-BP1
p70S6K1
ULK1
TFEB
Protein Synthesis
Autophagy
Synaptogenesis, Neuron survival, Learning/Memory
Mitochondrial dysfunction
Protein aggregation

Figure 7.14 mTORC1 pathway and regulation

mTORC1 is activated by insulin. Insulin/Akt signaling inhibits TSC1/2, thereby permitting the activation of the GTP-binding protein, Rheb. Rheb is the proximal activator of mTORC1. AMPK inhibits mTORC1 activity through indirect and direct mechanisms, phosphorylating TSC1/2 and the Raptor regulatory component of mTORC1. (Other trophic factors and pathways beyond insulin/Akt and AMPK, not shown for simplicity, also regulate mTORC1.) mTORC1 downstream targets include proteins involved the mRNA translation, 4E-BP1 and p70S6K1, and those involved in autophagy, such as the initiator of autophagy, ULK1, and the master regulator of lysosomal biogenesis, TFEB. By regulating the activity of these and other proteins, mTORC1 promotes protein synthesis, which is required for synaptogenesis, learning, and memory, but can also impair autophagy, leading to mitochondrial dysfunction and neurotoxic protein aggregation (Aβ, phospho-tau, α-synuclein, etc.). Black arrows and red lines respectively represent positive and negative regulation.

7.4.4 Antagonistic pleiotropy

"Antagonistic pleiotropy" is a term typically used to refer to an evolutionary tradeoff between fitness in early life at the expense of health later in life [466]. An example of antagonistic pleiotropy is the *ApoE4* allele, the leading genetic risk factor of AD [467]. This allele sensitizes the immune system and protected ancestral humans from infections that compromised reproductive fitness and cognition [468, 469]. Further relevant to modern contexts, *ApoE4* is associated with accelerated neurodevelopment [470] and improved memory during youth [471].

Another example of possible antagonistic pleiotropy in neurodegenerative disease is that of adiponectin (APN), a hormone secreted by adipose tissue. APN has broad beneficial functions on metabolism, including stimulating neurogenesis, and is generally thought to be neuroprotective [472, 473]. However, APN can induce astrocyte mediated neuroinflammation [318], oxidative stress [474], and plasma levels of APN are correlate with severity of cognitive decline and Aβ accumulation [475]. (For a review of antagonistic pleiotropy with regards to APN, see: [476]).

Hashimoto et al. have even proposed that amyloidogenic proteins, including Aβ in AD and α-synuclein in PD, might exhibit antagonistic pleiotropy. They hypothesize that the heterogeneity of amyloidogenic aggregates reflects the heterogeneity of metabolic stressors to which the human brain is exposed, and that specific amyloidogenic aggregates may serve to "precondition" the brain against future toxic exposures [477]. In effect, Aβ and α-synuclein could serve, in youth, as adaptive hormetic stressors. (As an aside, the Aβ/α-synuclein antagonistic pleiotropy hypothesis is intertwined with the "evolvability hypothesis" of amyloidogenic proteins, which is beyond the scope of this piece and reviewed by Hashimoto et al. [477]).

The moral of these examples – *ApoE4*, APN, and Aβ/α-synuclein – is that the trade of better health and cognition during youth, at the expense of cognition during non-reproductive years, was evolutionarily judicious. Furthermore, each these examples would not have been specifically mentioned if they did not plausibly involve mTORC1. With respect to *ApoE4*, mTORC1 activates pathways that promote synaptogenesis and neuronal development, which would benefit cognition during youth at the expense of decreased autophagy and increased risk of accumulating mitochondrial damage and neurotoxic protein aggregates over time, as in the case of *ApoE4* [470, 471]. Indeed, the *ApoE4* genotype is associated with elevated mTOR signaling [478]. APN has been shown to induce oxidative stress in an mTORC1-dependent manner by modulating both insulin and AMPK signaling [474] (Figure 7.14). And, of course, mTORC1 activity is assumed to be culpable for dysfunctional autophagy and accumulation of neurotoxic protein aggregates in neurodegenerative diseases, as noted in the introduction. Thus, mTORC1 may be a keystone player of antagonistic pleiotropy in neurodegenerative disease.

Consideration of antagonistic pleiotropy is important for evaluating the preventative value of inhibiting mTORC1 prior to the development of symptoms. No doubt, it's important to prevent the development of the pathologies underlying neurodegenerative diseases, which are established decades before symptoms develop [479-481]. But when and by how much? During mid-life, should one strive for mTORC1 inhibition, or value activating mTORC1 in a cyclic manner in order to build neural networks and increase her/his cognitive reserve, thus protecting against cognitive decline later in life? These are open questions.

7.4.5 Early/late-stage specificity

Although inhibiting mTORC1 to increase autophagy (and therefore clear damaged mitochondria and protein aggregates) may seem like a prudent intervention for neurodegenerative diseases, that may not be universally true. What if a disease progresses past a threshold beyond which the pathology is too well established to be meaningfully improved by an upregulation in autophagy? For instance, the mTORC1 inhibitor, rapamycin, does not reverse pathology or benefit cognition in late-stage AD models [482]. More importantly, because mTORC1 can inhibit apoptosis by activating p70S6K, which itself inhibits the pro-apoptotic protein BAD [483, 484], what if inhibiting mTORC1 beyond this hypothetical threshold increases apoptotic cell death?

Evidence consistent with this hypothesis is provided by multiple independent cell and rodent models of PD. These models of established late-stage disease suggest that increasing, rather that decreasing, mTOR activity could be beneficial under certain circumstances. In MPP^+-treated SH-SY5Y cells, activation of mTOR with cannabidiol led to protection against MPP^+-induced cell death [485]. In genetic and pharmacologic mouse models, upregulation of mTOR signaling (through PTEN ablation) is likewise associated with less cell death and improved symptomology [486]. A limitation of these early PD studies is that they do not involve α-synuclein accumulation, which may better recapitulate the human form of the disease and relative importance of autophagy therein. Nevertheless, given the knowledge that mTORC1 can inhibit apoptosis, and distinct possibility that there may be a point past which activation of autophagy is insufficient to improve disease course [482], it's worth questioning whether mTORC1 inhibition could actually be harmful in late-stage neurodegenerative disease.

7.4.6 Regional specificity

In addition to considering the temporal dimension (early/late-stage disease), it's important to consider the spatial dimension. As the brain is partitioned into networks, nuclei, and cell types, a given intervention may impact one region differently than another. For example, Ramalingham et al. discovered that rotenone injections (used to generate murine models of PD) oppositely impact mTORC1 activity in different regions of mouse brains, increasing activity in the midbrain and decreasing activity in the striatum [487]. Lifestyle interventions (more on this below), such as exercise, may also alter mTOR activity in a region-specific manner. In mice, wheel running regulates mTORC1 signaling most strongly in the nucleus accumbens and hippocampus, as compared to other brain regions [488]. This is notable because atrophy of nucleus accumbens and hippocampus is most strongly associated with AD [489].

The data are nascent but sufficient to issue caution. What if a PD patient suffering from substantia nigra atrophy were treated with an mTORC1 inhibitor based on a rationale from data collected from hippocampal pathology? What if a frontotemporal dementia (FTD) patient suffering from primarily temporal lobe pathologies was treated with an mTORC1-targeting drug based on frontal lobe data? As there is limited evidence to support that mTORC1 responds consistently to a wide range of interventions across brain regions, and some evidence to the contrary, it's responsible to not overgeneralize and assume globalized impact on the brain. More research needs to be conducted on the region-specific impacts of different mTORC1-directed interventions.

7.4.7 Disease specificity

While many neurodegenerative diseases share several key core pathologies, including mitochondrial dysfunction, protein aggregation, oxidative stress, and inflammation, it's also important to consider disease-specific aspects of neurometabolim that could interact with mTORC1. For example, Zhuang et al. recently discovered that TFEB activity (which stimulates lysosomal biogenesis and promotes autophagy) is increased in a 6-OHDA-treated SH-5YSY model of PD, as well as in dopaminergic neurons, and that TFEB activity is calcium/calcineurin-dependent [490]. This is important because PD is characterized by loss of substantia nigra pars compacta neurons, which exhibit a unique form of calcium pacemaking activity not seen in most other neurons. This suggests that regulation of autophagy may be different in the brain region most affected by PD as compared to brain regions impacted in other diseases.

Another example is Amyotrophic Lateral Sclerosis (ALS), which can be caused by loss-of-function mutations in the *UBQLN2/4* genes. While the products of these genes, ubiquilin proteins, are known best as components of the ubiquitin-proteasome system, they are also important in autophagy. Specifically, ubiquilins are required to maintain the vacuolar H^+-ATPase function that acidifies lysosomes [491]. In a scenario in which mTORC1 were inhibited to induce autophagy in ALS, induction of autophagy and lysosomal biogenesis may be increased (Figure 7.14), but if lysosomes are not sufficiently acidic to destroy the contents of the autophagosome, the contents could accumulate and exacerbate cellular stress. Therefore, inhibiting mTORC1 to upregulate autophagy could impair autophagic flux, leading to a back-up of components, and be harmful in such genetic cases of ALS.

7.4.8 Personalized therapy

There is a need for informed, disease-specific interventions. In this section, we provide three hypothetical examples of personalized interventions involving mTORC1. These will include glutamatergic antagonism for Huntington's disease (HD) [492], metformin treatment for multiple sclerosis (MS) [493], and SMCR8-centered therapy for ALS and FTD [494].

Glutamate hyperactivity plays a prominent role in HD [495] and can activate mTORC1 via the mGluR5-PDK1-Akt-mTORC1 pathway [492]. Correspondingly, Abd-Elrahman et al. recently demonstrated, in a mouse model of HD, that antagonism of the mGluR5 metabotropic glutamate receptor can correct overactive mTORC1 signaling and, consequently, increase autophagic clearance of mutant huntingtin protein. The authors of this paper also point out that huntingtin aggregates sequester the transcription factor, CREB, leading to a down regulation of neuroprotective BDNF. They show that mGluR5 inhibitors not only clear pathological aggregates, but also increase BDNF expression [492]. Therefore, mGluR5 antagonism, by inhibiting hyperactive mTORC1, could simultaneously promote the clearance of pathological huntingtin aggregates and increase neurotrophic factor signaling.

MS is characterized by demyelination of nerve cell axons. As oligodendrocytes are responsible for building myelin sheaths within the central nervous system, a goal of MS treatments is to boost oligodendrocyte renewal and remyelination. In a cuprizone-challenge mouse model of MS, Sanadgol et al. recently reported that the diabetes drug, metformin, did precisely that: it increased oligodendrocyte renewal and remyelination. These beneficial effects were mediated by a direct

stimulatory interaction between metformin and AMPK, and subsequent inhibition of mTORC1 [493] (Figure 7.14). Thus, metformin is a candidate for an mTORC1-targeting therapy for MS.

Mutations in the *C9orf72* gene are the leading cause of inherited ALS and FTD. Only recently was it discovered that another protein, SMCR8, complexes with the C9orf72 protein to form a heterodimer that negatively regulates mTORC1 activity [494]. Furthermore, a *SMCR8*-deficient mouse model recapitulates the *C9orf72*-deficient phenotype, leads to a decrease in C9orf72 protein, and is associated with upregulation of mTORC1 activity and decreased autophagy [494]. Future treatments for genetic causes of ALS and FTD might consider SMCR8 therapy or other interventions that target the SMCR8-mTORC1-autophagy axis.

These examples were chosen because HD, MS, ALS, and FTD are lesser studied than AD and PD. However, the same personalization principle applies to all conditions in which mTORC1 plays a role. In PD, for example, levodopa-induced dyskinesia is thought to be induced by D1-receptor-mediated phosphorylation of mTORC1, a hypothesis supported by the fact that genetic variability in mTOR pathway components is associated with PD dyskinesia [496]. The development of useful future interventions for neurodegenerative disorders would benefit from a deeper consideration of the interactions between mTORC1 signaling and disease/patient-specific mechanisms.

7.4.9 Lifestyle interventions

Two reasons most neurodegenerative diseases are refractory to treatment are that interventions may be initiated too late in the disease process and/or are too specific. These limitations are a function of the pharmacologic approach to neurodegenerative disease in which symptomatic patients, who have usually been afflicted by the underlying disease for years to decades, are prescribed drugs not available for prevention during the preclinical stage. Certainly, drugs have their place. But to quell the neurodegenerative disease pandemic will require universally accessible preventative measures based on safe lifestyle interventions, including diet and exercise. Evidence suggests such interventions could operate, in part, through mTORC1-mediated mechanisms.

Turmeric is the best-studied nutraceutical for neurodegenerative diseases. In a genetic mouse model of AD, turmeric's active component, curcumin, inhibited mTORC1 to increase autophagy and prevent Aβ accumulation [497]. Correspondingly, curcumin-induced inhibition of mTORC1 protected against memory impairments in this model [497]. A more specific dietary example would be the mineral manganese in HD. As manganese deficiency might contribute to the pathogenesis of HD by affecting the insulin/Akt/mTORC1 pathway, correcting a simple micronutrient deficiency could be protective in some cases of HD [498]. A third example is that of PPARs, a family of transcription factors that can inhibit mTORC1 and promote autophagy to protect against neurodegenerative disease [430, 499]. Many nutrients and their derivates activate PPARs, including oleoylethanolamide derived from oleic acid in olive oil [500, 501] and the monoterpenes carvacrol and thymol found in mint family plants (basil, mint, rosemary, sage) [502, 503]. Curcumin, manganese, and dietary PPAR activators are just three examples of nutraceuticals

from different classes that, when combined in a well-formulated diet and with other dietary mTOR regulators [502, 504], could have a meaningful impact on cognitive longevity.

In addition to nutraceuticals and micronutrients, shifts in macronutrient intake can also impact mTORC1 activity. The most evident examples are intermittent fasting and high-fat, low-carbohydrate ketogenic diets, which can modulate mTORC1 activity through at least three mechanisms. First, fasting and ketogenic diets diminish insulin-mediated mTORC1 activation. Second, they activate AMPK (by altering the AMP/ATP ratio and causing glycogen depletion) to inhibit mTORC1 and induce autophagy [100, 505]. Third, fasting and ketogenic diets share the common feature of stimulating hepatic production of the ketone body, β-hydroxybutyrate, which itself is a signaling molecule that regulates mTORC1 [30, 506, 507]. Interestingly, it has recently been demonstrated that both short-term ketogenic diets and acute administration of exogenous β-hydroxybutyrate improve a marker of brain aging called "brain network stability," in contrast to standard Western diets and sugar which decrease network stability [508]. Long-term prospective studies will need to be conducted to determine whether fasting and ketogenic diets are truly neuroprotective in humans. Nevertheless, these mechanisms and data coincide with the growing popularity of intermittent fasting and ketogenic diets as prevention or treatment strategies for neurodegenerative conditions [30, 242, 508-516].

Exercise is another lifestyle intervention that benefits brain health. Prospective cohort and randomized controlled studies have found that exercise reduces the risk of developing dementia by as much as 38% [517] and improves cognitive function in those already living with AD [518, 519]. Kou et al. recently published a compelling review arguing that the benefits of exercise on

cognitive function and AD may be mediated by mTORC1 regulation [520]. Even a cursory consideration of this hypothesis suggests it has merit. Exercise alters nutrient flux, trophic factor signaling, and can activate AMPK. Exercise can also correct overactive mTORC1 signaling to increase autophagy by correcting dysfunctional microRNA expression in a mouse model of AD [521, 522]. These particular studies focus on microRNA-34a, but there is reason to believe that exercise can influence mTORC1, autophagy, and cognitive aging by regulating a wide network of microRNAs [520]. In another rodent model of AD, treadmill exercise decreased phospho-mTOR levels (Ser-2,448, Akt target residue [523]), increased autophagy, and completely rescued cognitive function on the Morris water maze test [524].

Dietary micronutrients, fasting and ketogenic diets, and exercise are but a few illustrative examples of lifestyle interventions that may interact with mTORC1 to modulate the course of neurodegenerative diseases. Additional therapies include probiotics to modulate the gut-brain axis, which has been heavily implicated in the development of neurodegenerative diseases [525, 526], and heat therapy to induce chaperone heat shock proteins (whose expression is at least partially mediated by mTORC1 [527]) that could promote the proper folding of amyloidogenic proteins [528-530]. At the present time, clinical studies examining the impact of lifestyle interventions on mTORC1 signaling for cognitive decline are few [520, 531] and more research needs to be conducted in this area to inform holistic and universally available best practices for the treatment and prevention of neurodegenerative disease.

7.4.10 Conclusions

While references to the most pressing open questions are scattered throughout the abundant literature on mTOR and neurodegenerative disease, herein, we have consolidated these gaps in the literature (Figure 7.15). How do we balance the beneficial effects of mTORC1 against its negative effects? How does this balance shift with disease progression or brain region? How can we use knowledge of biochemical pathways, specific to diseases and even individual cases, to inform personalized therapy? And what universally available lifestyle interventions might help in the prevention of neurodegeneration? Consideration of these mTOR mysteries will inform future research.

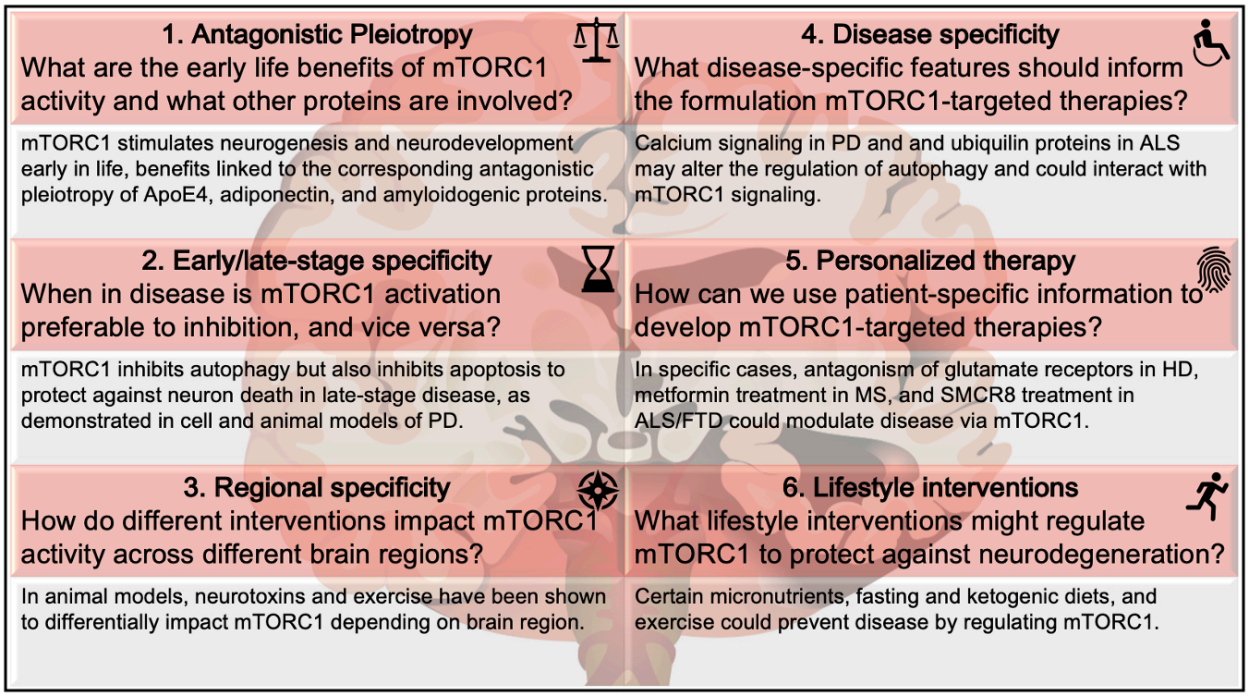


Figure 7.15 mTORC1 mysteries

Six nuances regarding mTORC1 in neurodegenerative disease. The questions and examples below each topic are illustrative, not comprehensive, of the literature covered in this review. Disease abbreviations: ALS, Amyotrophic Lateral Sclerosis; FTD, frontotemporal dementia; HD, Huntington's disease; MS, multiple sclerosis; PD, Parkinson's disease.

Chapter 8. *Case report and clinical suggestions for assessing cardiovascular risk for persons on ketogenic diets (peer-reviewed and published)*

Declaration

I certify that the contents of this thesis chapter represent my own ideas, work, and words. The second author provided data for the associated manuscript but was not involved in its composition.

8.1 **Norwitz NG**, Loh V. A standard lipid panel is insufficient for a patient on a high-fat, low-carbohydrate ketogenic diet. *Frontiers in Medicine*. 2020

8.1.1 Statement of relevancy

The perception that high-fat diets are intrinsically bad for cardiovascular health presents a major obstacle to the clinical implementation of ketogenic diets for neurodegenerative and neurological diseases. The origin of the belief that high-fat diets, and particularly those high in saturated fat, are bad for heart health is derived from the assumption that high-fat diets can increase levels of LDL cholesterol. Indeed, it has been anecdotally reported that ketogenic diets can increase LDL; however, *how* they do so is important:

As opposed to high-fat "Westernized" diets, which should more accurately be called high-carbohydrate and high-fat diets, ketogenic diets are nearly devoid of carbohydrates. In the case of high-carbohydrate and high-fat diets, LDL cholesterol increases because carbohydrates can lead to the glycation of the ApoB lipoprotein on LDL particles, preventing LDL particle reuptake by the liver and increasing their half-life in the blood. As a result, younger and larger LDL particles can condense and become oxidized and form small dense LDL, which is atherogenic.

By contrast, ketogenic diets can increase LDL levels via carbohydrate restriction. Triglycerides are shuttled by large LDL particles to peripheral tissues. Therefore, a metabolic shift from a carbohydrate dependence to fat dependence might logically induce an increase in young, large LDL secretion. Provided these large LDL particles are not glycated and oxidized, which is less likely in the absence of dietary carbohydrates, they can function to transport triglyceride fuel before being resorbed by the liver and prior to condensing and becoming atherogenic. Therefore, on ketogenic diets, LDL particles may increase because of a beneficial adaptation and this may not represent an atherogenic insult.

Unfortunately, standard lipid panels, which include total cholesterol, LDL-C, HDL-C, and triglycerides, cannot distinguish between the type of LDL increases seen on standard high-carb and high-fat diets and those hypothesized to occur on ketogenic diets. Furthermore, no longitudinal interventional or observational clinical trials have specifically looked in-depth at how cardiometabolic markers change following adaptation to a ketogenic diet.

The short comings of the literature, and the negative effect this might have on the care of patients with neurodegenerative and neurological diseases who might benefit from ketogenic therapy, inspired this report.

This manuscript represents a first-of-its-kind in-depth investigation of how lipids and cardiometabolic markers change when an individual adopts a therapeutic ketogenic diet. The findings match the prediction of the above model and suggest that changes that would have been

marked as adverse on a standard lipid panel (and compelled the subject to abandon his therapeutic ketogenic diet) were neutral to beneficial.

The main limitation of this manuscript is that it is based on only a case report. However, it is the first publication to provide direct evidence that standard lipid panels may be insufficient for the care of individuals on ketogenic diets. Furthermore, it was intentionally written the manuscript as a mixed-manuscript – a combination of case report and review of the literature – in the hopes that it could be used as an instructional tool for interested clinicians and that it could serve as a stepping-stone towards the commencement of proper clinical trials investigating the same question in larger and more heterogenous cohorts of patients.

(The attention this manuscript received upon publication, including being listed in the top 1% of popular publications from a database of over 15 million based on Altmetric score, hints that it was successful in its goal to initiate an important conversation.)

In brief, this publication serves as a first step towards reexamining how cardiovascular risk is assessed for patients on ketogenic diets. It therefore could help to remove an obstacle to the clinical implementation (and optimization) of ketogenic diets for neurodegenerative and neurological diseases, enabling the broader use of this metabolic tool to address patients' symptoms and disease progression.

8.1.2 Abstract

High-fat, low-carbohydrate ketogenic diets have recently become popular for weight loss and the treatment of numerous chronic diseases; however, the general medical community still expresses concern regarding the impact of high-fat diets on serum lipids and cardiovascular risk. Herein, we report on a young man who adopted a ketogenic diet to treat his inflammatory bowel disease. Incidentally, changes in his serum lipids that would be considered adverse by current standards were noted. A more critical analysis of his lipid profile suggests that the changes he experienced may not be dangerous and may, at least with regard to several parameters, represent improvements. This case study demonstrates how the manner in which lipid panels are often reported and reviewed can lead to misleading conclusions and highlights that, at least in the care of those on a ketogenic diet, more nuanced analyses of lipid subfractionations should be conducted in order for physicians to provide optimal care and clinical recommendations.

8.1.3 Introduction

Ketogenic diets are high-fat, low-carbohydrate diets that induce the liver to generate ketone bodies, particularly the ketone body β-hydroxybutyrate, a metabolic energy source and signaling molecule evolutionarily designed to efficiently fuel the brain and body during times of carbohydrate scarcity. Although clinical studies implementing ketogenic diets have heretofore been difficult to conduct, and more research is needed, a body of data demonstrates that ketogenic diets may be useful in the treatment of a wide range of chronic diseases that share inflammation as a common underlying pathology [245, 532-537]. One of these diseases is ulcerative colitis, an inflammatory bowel disease. Within the gastrointestinal tract, ketogenic diets may dampen inflammation by inhibiting activity of the NLRP3 inflammasome [248], promoting intestinal stem cell regeneration and gut healing [538], and stimulating the release of bile acids that facilitate intestinal immune system homeostasis [539, 540].

Because ketogenic diets are high-fat, and the still-prevalent lipid heart hypothesis assumes high dietary fat intake causes poor cholesterol profiles and elevated cardiovascular risk, it is medically responsible to follow the serum lipids of individuals who consume ketogenic diets. How rigorously we follow serum lipids matters. Standard lipid panels commonly ordered by practitioners typically only report total cholesterol, HDL-C, LDL-C, and triglycerides. These simple metrics are then used to make clinical recommendations.

However, it is now well-established that lipoprotein particles display remarkable heterogeneity in form and function. For example, larger LDL particles appear to be nonatherogenic [541, 542] and different HDL particles secreted by the liver appear to display different antiatherogenic properties

in association with their distinct morphologies [543, 544]. Furthermore, other lipid parameters highly relevant to cardiovascular risk, such as oxidized LDL and Lp(a) measurements, are not typically reported. The standard practice of ordering a simple total cholesterol, HDL-C, LDL-C, and triglycerides panel is likely rationalized by the assumption that there is enough homogeneity in the population for these basic measurements to provide sufficient information to make clinical recommendations. This case demonstrates such assumptions may not hold true, particularly in those practicing high-fat, low-carbohydrate ketogenic eating.

8.1.4 Case description

The subject is a 24-year-old white Caucasian male with biopsy-confirmed ulcerative colitis, diagnosed at age 21. He exhibits normal blood pressure and a healthy BMI, leads an active lifestyle, and has no history of relevant comorbidities (including diabetes or pre-diabetes), nor confounding lifestyle patterns (including smoking or alcohol abuse). From the time of his diagnosis, his ulcerative colitis has been his chief medical complaint. Although his inflammatory bowel disease temporarily went into symptomatic remission with oral mesalamine and prednisone, he began to experience repeated flares several months later. He was subsequently treated with mesalamine and prednisone enemas, both of which also failed to induce remission for more than three months. A total of three colonoscopies, as well as measurements of fecal calprotectin, revealed persistent rectosigmoid inflammation. The subject also experienced other inflammatory phenomenon during this time, including swollen joints and rosacea, although his hsCRP remained below 3 mg/L. In addition to his prescription medications, the subject adopted and strictly adhered to several diets, including the popular low FODMAP and specific carbohydrate diets, none of which put his disease into lasting remission. At age 23, still with the aim of finding a diet that

would ameliorate his gastrointestinal symptoms, the subject adopted a ketogenic diet on his own initiative, with 75-80% of his daily calories derived from fat (~300 grams), 15%-20% derived from protein (~130 grams), and 4-5% derived from carbohydrates (~30 grams). His self-reported major fat, protein, and carbohydrate sources highlighted extra virgin olive oil and avocados, seafood, and low-carbohydrate, high-fiber vegetables. Within one week of adopting this Mediterranean-style ketogenic diet, his gastrointestinal symptoms improved and his fecal calprotectin dropped from 123 μg/g to 19 μg/g, which is within the normal range of <50 μg/g. At the time of this writing eight months later, the subject has not experienced another colitis flare, his compliance has been monitored by periodic assessment of his blood β-hydroxybutyrate levels, which range between 1.0 mM and 3.0 mM, and his calprotectin, remeasured on three separate occasions, has remained within the normal range, despite the fact that his has discontinued all of his prescription medications for ulcerative colitis.

Given the high-fat nature of the subject's diet, his serum lipids were closely followed. One week prior to starting the diet, a subfractionated lipid panel was drawn and was repeated seven months later. Several alarming changes in conventional lipid parameters were flagged in the report in red, as follows: His total cholesterol increased from 160 mg/dL to 450 mg/dL; his LDL-C increased from 95 mg/dL to 321 mg/dL; and his LDL-P increased from 1143 to 2259 (Figure 8.1A). These seemingly adverse and dramatic changes were in small part offset by an improvement in his HDL-C from 48 mg/dL to 109 mg/dL.

Since total cholesterol, HDL-C, and LDL-C are the cholesterol parameters reported in a standard lipid panel, these are the measures that would typically be used to direct this subject's care. Indeed,

according to modifiable risk factor scoring criteria similar to those derived from Framingham, but established for individuals ages 15 – 34, these changes ostensibly represent a 4-fold increase in the subject's risk for atherosclerosis [545]. However, in this subject, a full fractionated panel was pursued and additionally included a size-based breakdown of the subject's LDL and HDL lipoprotein particles, LDL and HDL particle counts, Apo(B) mass, oxLDL, Lp(a), and PL-PLA2 activity (Figure 8.1A-B).

Of note, at the time of the seven-month follow-up, a coronary artery calcium scan was also performed as a functional assay of plaque formation. It revealed no significant findings and reported a score of 0, suggesting no signs of calcified atherogenic plaques (Figure 8.1C).

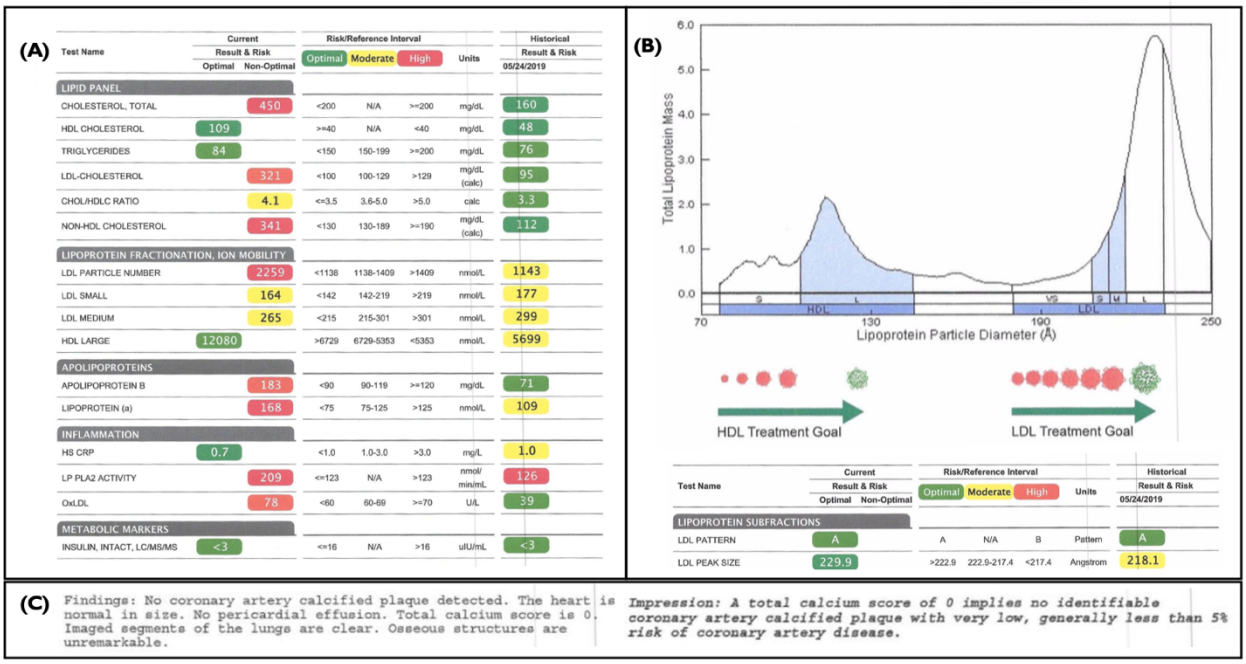


Figure 8.1 Lipid subfractionation

(A) The subject's baseline lipids, prior to starting his ketogenic diet, are shown on the right. His 7-month follow-up lipids are shown on the left. Green, yellow, and red imply optimal, medium, and high cardiovascular risk, respectively, based on standard reference ranges of isolated variables. (B) The subject's lipoprotein size distribution is characterized by a multimodal distribution of HDL, with the greatest peak being in large HDL, and a strong bias in LDL away from atherogenic, small LDL and toward large LDL. (C) Results of the subject's coronary artery calcium scan, performed at the time of the 7-month follow-up, indicating little-to-no calcified plaque accumulation.

8.1.5 Discussion

A standard lipid panel would have revealed that, in this subject, a ketogenic diet induced a large increase in total cholesterol and LDL-C, shifting both parameters from the optimal range to more than twice the threshold for the high-risk range. Even on the subfractionation, the visuals of the report itself indicated cause for clinical concern: an overwhelming shift in color parameters from normalizing green/yellow to alarming yellow/red (Figure 8.1). Therefore, it is likely that, were this subject only analyzed by a standard lipid panel, or were the results of the subfractionation not holistically and critically scrutinized, the clinical recommendation would have been that the subject cease his ketogenic diet, a presumptive agent of his colitis remission. Furthermore, this subject may also have been prescribed a statin at 24-years-old. Given the mixed evidence on the use of statins for primary prevention and the possibility that long-term statin use in low-risk individuals (including those with a coronary artery calcium score of 0) can contribute to atherosclerosis [546], such a recommendation could have had negative long-term cardiovascular health consequences.

What follows is a more nuanced analysis and discussion of the most relevant changes in the subject's lipid panel, which we suggest do not confer as large an increase in risk as a cursory analysis of certain isolated measures, and the report's color scheme, otherwise imply. An argument could even be made that these changes represent an improvement.

We will start with total cholesterol change and HDL cholesterol as a partial contributor to that change. It is notable that the subject's total cholesterol almost tripled from 160 mg/dL to 450 mg/dL. Although remarkable, this change alone is not informative without a further consideration of what particles are driving the change. A substantial minority of the increase was driven by a doubling of the subject's HDL cholesterol from 45 mg/dL to 109 mg/dL. Since HDL has antiatherogenic functions, including not only reverse cholesterol transport, but also antioxidant and anti-inflammatory properties, this change is somewhat reassuring. Furthermore, is it generally agreed that, while high HDL-C is associated with good cardiovascular health, HDL-P is a superior predictive measure [543, 544, 547]. The subjects large HDL-P jumped from 5,699 nmol/L to a remarkable 12,080 nmol/L. Nearly a dozen separate studies suggest that large HDL particles have a particularly strong association with low cardiovascular risk, even as compared to smaller or medium HDL particles [548-558]. However, while there is some disagreement in the field about which HDL particles (small, dense, large, or buoyant) are the most cardioprotective, it has been proposed that it a mix of HDL particles with different morphologies may be ideal [543, 559]. This is because, unlike LDL particles, which are secreted in a single form from the liver and decay in size over time, HDL particles are secreted in different forms by the liver and these different forms likely have different functions [559]. For example, larger HDL particles may have greater antioxidant capacity, whereas small dense HDL3c may be particular efficient at reverse cholesterol transport [543, 544]. Based on the probable correlation between HDL particles' diverse forms and functions, and the epidemiological data, one could argue that an ideal HDL profile would display a multimodal distribution, one with an overall high particle count with the greatest peak in large HDL-P. This is precisely what is observed in this subject (Figure 8.1B).

Next, we can examine LDL cholesterol as a major contributor to the subject's total cholesterol increase. Between baseline and follow-up, the subject's LDL-C increased from 90 mg/dL to 321 mg/dL, the former measure being marked as optimal, and the latter, in alarming red, being twice the threshold of high-risk (Figure 8.1A). However, not all LDL particles are equal. The association between LDL-C and cardiovascular risk is driven by the association between LDL-C and atherogenic small dense and/or oxidized LDL [541, 542]. It is primarily the small dense and/or oxidized LDL particles that can penetrate the endothelial wall, be taken up by circulating macrophages, and contribute to foam cell and plaque formation [560, 561]. Large LDL particles, by contrast, do not display an association with cardiovascular risk, and may, in fact, be cardioprotective [542, 562]. A review of the subject's change in LDL-P (from 1143 to 2259) and size-based LDL subfractionation reveals that the increase in his LDL is driven exclusively by an increase in large LDL. Both his small and medium LDL even exhibited decreases of 8 and 11%, respectively (Figure 8.1A).

Since the biological function of LDL is, at least in part, to carry triglycerides from the liver to peripheral tissues as a source of fuel, it is not at all surprising that the subject exhibited an increase in large LDL given his high-fat diet. Furthermore, the fact that only his large LDL increased suggests the subject's large LDL particles did not tend to decay overtime into medium and small LDL. Stated more directly, the subject's specific increase in large LDL is consistent with an increase in LDL turnover rate and liver uptake.

This represents a positive and adaptive response to the subject's switching from carbohydrate-based metabolic fuels to fat-based metabolic fuels. This analysis and discussion of LDL metabolism also explains why the increase in LDL-P and Apo(B), both driven by an increase in large LDL-P, may likewise represent healthy and positive adaptions.

Thirdly, we can consider a lesser-known and studied lipoprotein particle, Lp(a). Structurally, Lp(a) is highly similar to LDL except that, appended to Apo-B100, it possesses a glycoprotein tail, apolipoprotein (a). Apolipoprotein (a) itself is remarkably similar in sequence and form to plasminogen, the enzyme that, when activated, binds to and degrades fibrin to break up blood clots. However, apolipoprotein (a) lacks the same protease activity as plasminogen. Lp(a) can thus compete with plasminogen for fibrin binding (inhibiting fibrinolysis) and contribute to the formation of endothelial clots, i.e. atherogenic plaques [563]. It therefore makes sense that Lp(a) levels correlate positively with cardiovascular risk [564].

Another role for Lp(a) has also been hypothesized, following on the observations that 90% of oxidized lipoproteins (oxLPs) bound to ApoB-containing lipoproteins are actually bound to Lp(a) and that LP-PLA2, an enzyme that degrades atherogenic oxLPs, is associated with Lp(a). It has been proposed that Lp(a)-LP-PLA2 acts as a scavenger for oxLPs [565]. Therefore, the subject's increase in Lp(a) and LP-PLA2 activity could both be adaptive responses to the increase in his oxidative status, marked by the increase in his oxLDL (Figure 8.2).

To interject an added nuance, before returning to the topic of the subject's oxLDL, it possible that the his elevated LP-PLA2 activity does not represent a risk at all because (i) on Apo-B-containing particles, LP-PLA2 is most active on small LDL [566, 567] and (ii) HDL-associated LP-PLA2 activity may be antiatherogenic [565, 568, 569]. Therefore, this subject's low small-LDL-to-HDL-particle ratio may conceal a cardioprotective factor behind the guise of an atherogenic one.

The increase in the subject's oxLDL is the single change that, in our opinion, is most probably negative. Given that the shift in the subject's diet included an increase in his intake of oxidation-prone polyunsaturated fats (in the form of nuts, seeds, and fatty fish) and a decrease in his intake of antioxidant-containing produce (including vitamin C-containing citrus fruits) it is not entirely surprising that his oxLDL increased. We remark specifically on vitamin C as an antioxidant because another proposed function of Lp(a) is as a surrogate to vitamin C [563]. This hypothesis stems from the observations that (i) animals that produce vitamin C endogenously tend not to possess Lp(a) or exhibit heart disease, (ii) there in an inverse correlation between vitamin C status and Lp(a) levels, and (iii) vitamin C is essential in the process of collagen synthesis and endothelial repair. Thus, an evolutionarily adaptive response to insufficient vitamin C would be to increase levels of an antifibrinolytic factor, Lp(a), to induce clot formation and prevent excess bleeding [563].

We therefore hypothesize that, were this subject to increase his intake of low-carbohydrate vitamin C-containing foods (such as strawberries, bell peppers, broccoli, and cauliflower) and/or supplement with vitamin C, and also decrease his intake of polyunsaturated fats, exchanging them for more oxidation-resistant monounsaturated fats and possibly some saturated fats that are less

likely to impact LDL (including virgin/raw coconut products and stearic acid-rich cacao), he would display a decrease in oxLDL and a consequent decrease in Lp(a) and LP-PLA2 activity (Figure 8.2). These clinical recommendations have been made but the subject of this study has relocated and is not currently available for follow-up. We are, however, collecting to-be published longitudinal data on several other patients undergoing vitamin C treatment for elevated Lp(a).

Our report has several limitations. First, no serum cytokines (such as TNF-α and IL-1β) or serum endotoxin were tracked in this patient. It would have been informative to document whether his ketogenic diet improved these markers despite his consistently low hsCRP, as has been reported in other patients adopting ketogenic diets [534]. In addition, it is unfortunate that this subject is not currently available for follow-up to document whether the recommended adjustment to his diet altered his, Lp(a), LP-PLA2 activity, and oxLDL levels, as we hypothesized. Nevertheless, our analysis of this subject's subfractionation, in conjunction with the functional observation that his coronary artery calcium score is 0, indicating no atherosclerotic plaque formation, argue the changes is his lipids may not be negative, but rather positive.

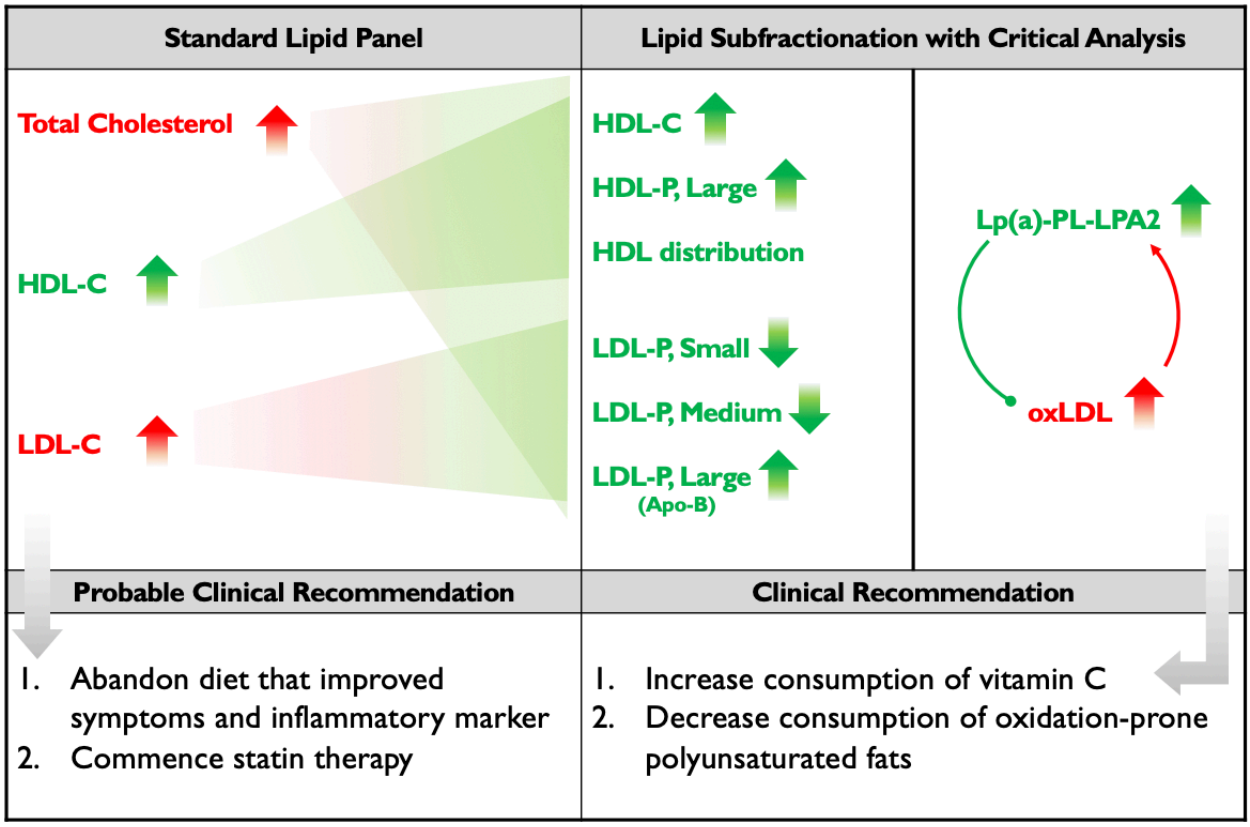


Figure 8.2 Standard lipid panel vs. lipid subfractionation

The left column denotes changes induced by the subject's diet that would have been detected on a standard lipid panel, along with the probable course of clinical action. The middle-right column denotes the changes that were detected by subfractionation and, below, the clinical course of action that was recommended. Red indicates changes presumed to be negative; green indicates changes presumed to be positive. Apo(B) is placed in parentheses, below large LDL, because the subject's increase in Apo(B) mass was driven by his increase in large LDL. The diagram within the far-right column illustrates the hypothesis that the subject's elevated Lp(a) and LP-PLA2 activity may represent adaptive, protective responses to the increase in his oxLDL.

8.1.6 Summary and significance

Herein, we report on a subject who adopted a ketogenic diet for ulcerative colitis that successfully put his condition into remission but was also associated with an ostensibly adverse change in his serum lipid profile. A deeper analysis of these lipid profile changes reveals that many parameters may, in fact, be positive. Therefore, rather than recommending the subject abandon the diet that has proved successful in treating his disease, we have recommended a slight nutritional adaptation to see if that optimizes his lipid profile and health.

The significance of this report is three-fold: (i) Although clinical anecdotes reveal that ketogenic diets can improve the symptoms of patients struggling with inflammatory bowel diseases, there is little published data on the topic (possibly owing to the high variability among human microbiomes and, thus, patient responsiveness). This report documents an instance in which a ketogenic diet clearly improved a patient's colitis symptoms and laboratory inflammatory markers. (ii) We herein provide data that strongly suggest, at least in the case of subjects on ketogenic diets, standard lipid panels may not be sufficient, and that analyses of lipid subfractionations may be required, in order to inform optimal clinical recommendations. In closing, (iii) this case represents an example of the positive trend in medicine away from formulaic care and toward holistic, personalized, and integrative care.

Chapter 9. *Summary*

This DPhil was originally intended to include three interventional clinical studies in which ketone ester (KE) was administered to persons with Parkinson's disease (PD). Two studies are complete and the last was successfully setup and initiated. Study 1 was a placebo-controlled crossover trial with 14 participants to test whether KE supplementation could improve endurance exercise performance. It yielded positive results on the primary outcome variable. On average, participants sustained the target cadence for significantly longer in the KE group as compared to the isocaloric carbohydrate placebo, supporting the notion that KE may operate as an adjunctive therapy to improve therapeutic exercise treatment for PD.

Study 2 examined whether KE supplementation altered brain metabolism in 11 participants with PD by comparing fasted baseline ^{31}P-MRS scans to scans taken one hour after drink consumption. No changes in ATP, NAD(H), or any other measured cerebral metabolite were observed, although this does not preclude the possibility that chronic KE administration could be neuroprotective.

Study 3 aimed to investigate whether four times daily KE ingestion over 28 days could improve the symptoms of PD better than placebo. Study setup, including ethical approval and all contracts, is complete and four participants were completed through the intervention prior to study suspension forced by the pandemic. Study 3 is included herein as a feasibility and validation of methods.

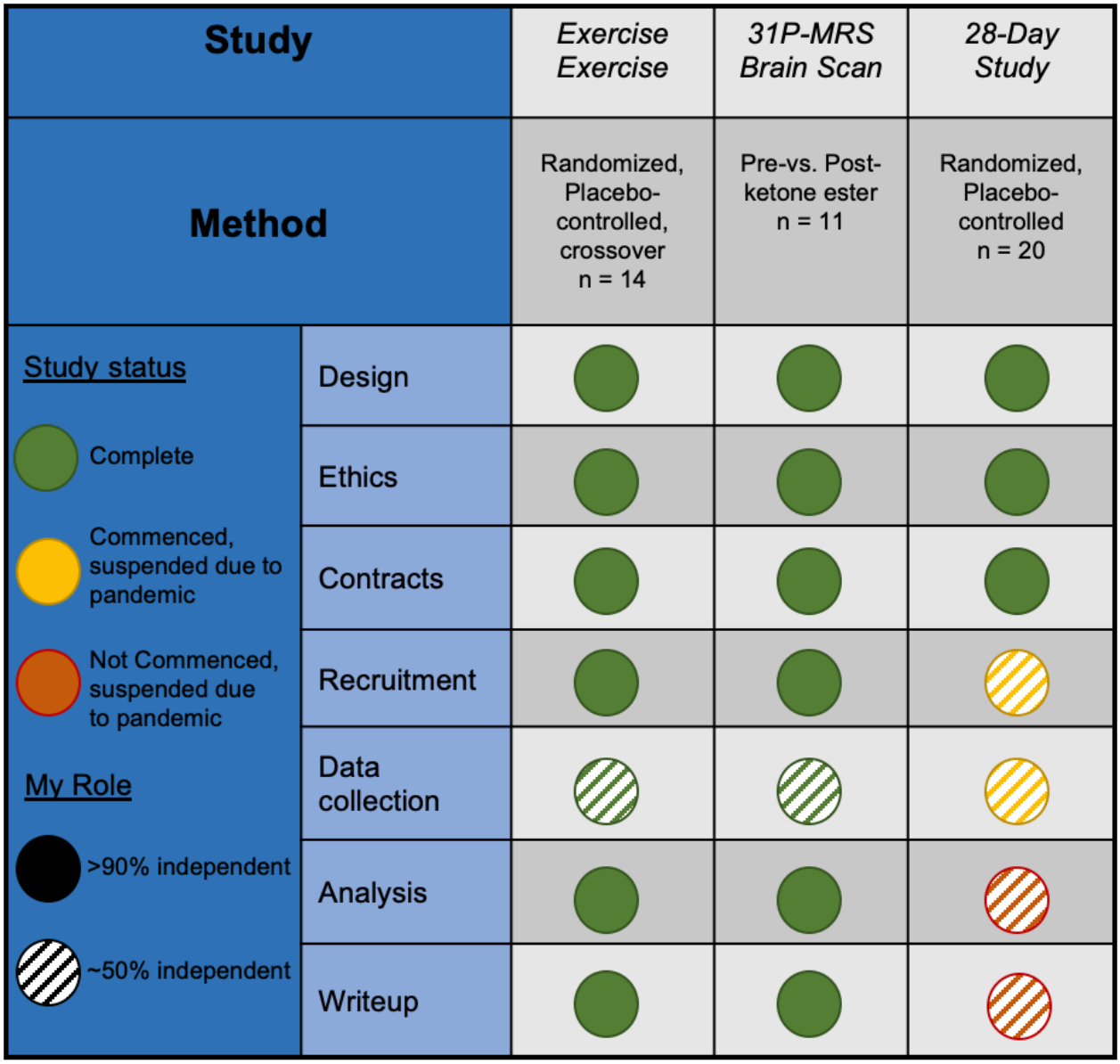
To supplement the data absent from the one incomplete PD study, this thesis includes other contributions to the fields of ketone metabolism and neurodegeneration research in the form of published manuscripts and an original nutrition tracking tool. The manuscripts are literature reviews, perspectives and theory papers, and an original case report and reappraisal of cardiovascular risk assessment for individuals on ketogenic diets. The nutrition tracking tool – MUNCH – is intended to open up avenues in nutrition research by enabling the conduct of accurate, long-term, and cheap human dietary studies. A list of accomplishments related to ketogenics and neurodegenerative and neurological diseases is presented in Appendix 8.

The contents of this work are united by the theme that neurodegenerative and neurological diseases are metabolic diseases that invite complementary metabolic treatments and, therefore, that ketogenics and nutrition may be used to address the root causes of these diseases. While it was not within the scope of a DPhil to evaluate the disease-modifying potential of ketogenics for neurodegenerative and neurological conditions in long-term clinical studies, this thesis contains evidence that exogenous KE supplementation can impact symptoms and offers rationales for why and how ketogenics and nutrition could provide disease-modifying options for patients where few currently exist.

Appendices

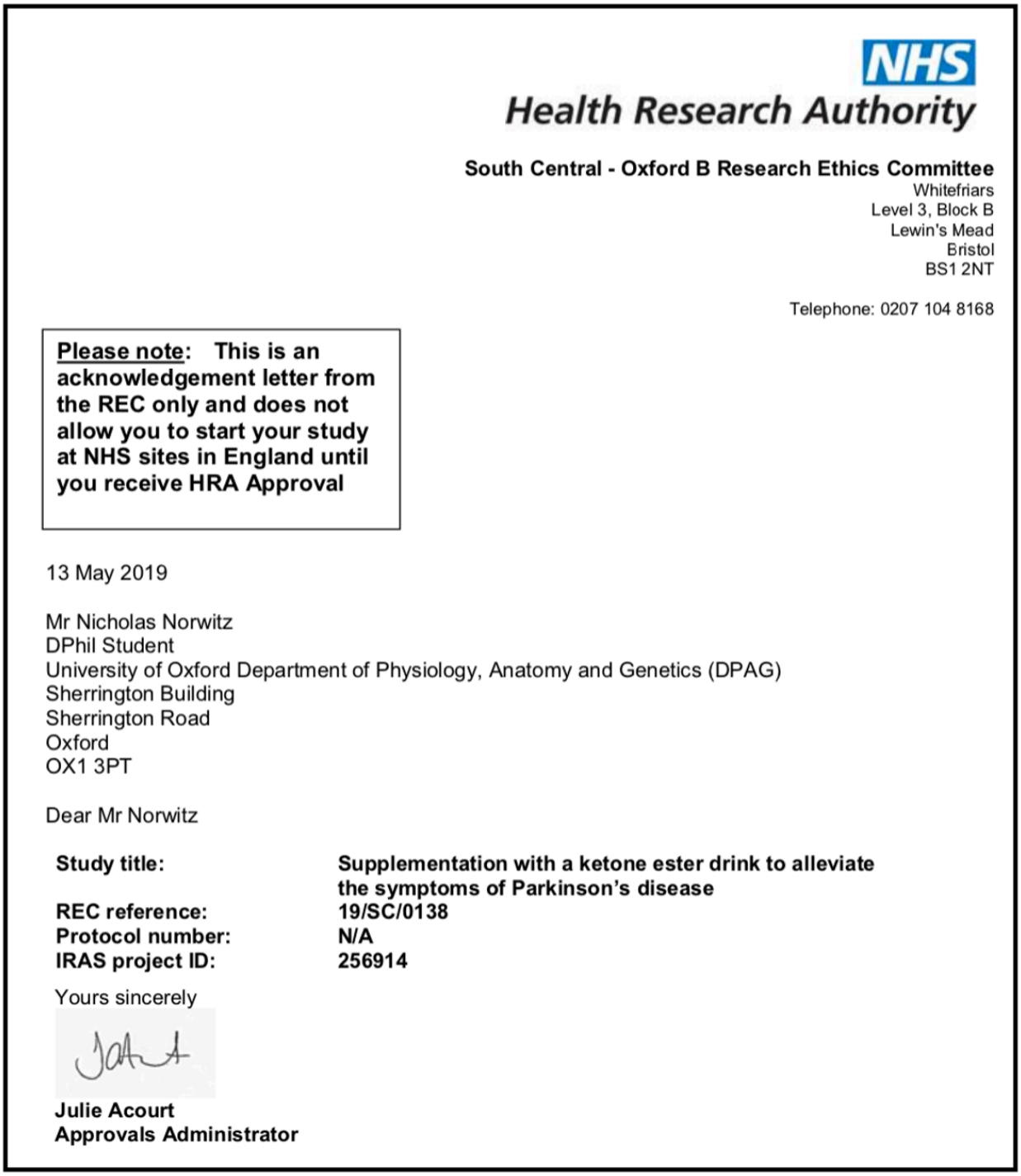
Appendix 1 Status of ketone ester in Parkinson's disease clinical studies

This dashboard is meant to visually clarify the impact of COVID-19 on the three clinical studies with ketone ester in Parkinson's disease, as well as the degree to which various tasks were completed mostly independently or with some support.



Appendix 2 REC and HRA approval samples

Research Ethics Committee (REC) and Health Research Authority (HRA) approvals were required for each of the three clinical studies that are the topic of chapters 3 – 5. Samples of the covers of those letters are shown below. The samples are for IRAS 256914, REC reference 19/SC/0138. Similar approvals were obtained for references 19/SC/0032 and 19/SC/0033.



NHS
Health Research Authority

South Central - Oxford B Research Ethics Committee
Whitefriars
Level 3, Block B
Lewin's Mead
Bristol
BS1 2NT

Telephone: 0207 104 8168

Please note: This is an acknowledgement letter from the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

13 May 2019

Mr Nicholas Norwitz
DPhil Student
University of Oxford Department of Physiology, Anatomy and Genetics (DPAG)
Sherrington Building
Sherrington Road
Oxford
OX1 3PT

Dear Mr Norwitz

Study title:	**Supplementation with a ketone ester drink to alleviate the symptoms of Parkinson's disease**
REC reference:	**19/SC/0138**
Protocol number:	**N/A**
IRAS project ID:	**256914**

Yours sincerely

Julie Acourt
Approvals Administrator

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor Michele Hu
Associate Professor and Honorary Consultant Neurologist
Oxford University Hospitals NHS Trust and Nuffield Department of Clinical Neurosciences, University of Oxford
Department of Neurology, West Wing, Level 3
John Radcliffe Hospital, Headley Way
Oxford
OX3 9DU

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

13 May 2019

Dear Professor Hu

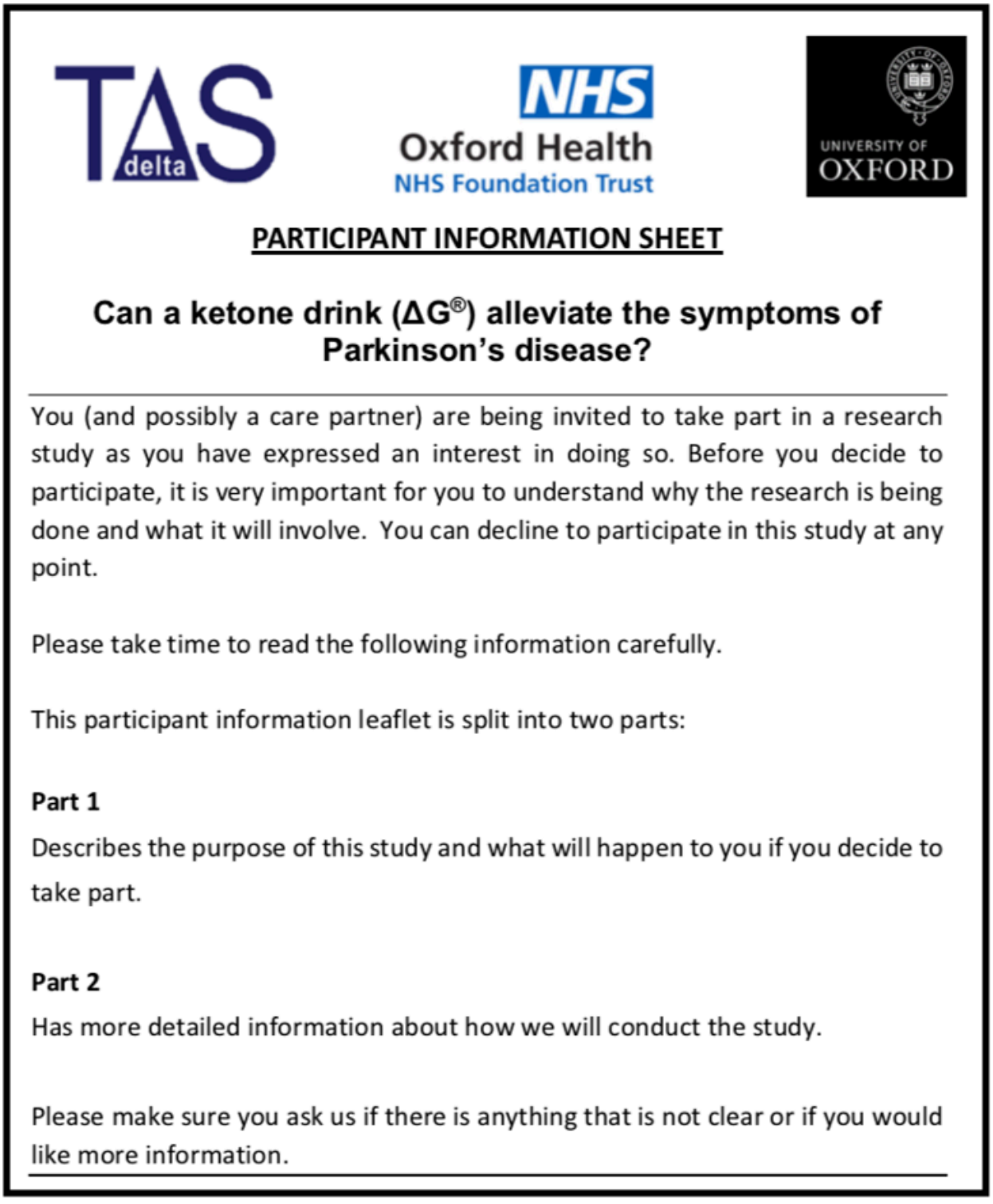
HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Supplementation with a ketone ester drink to alleviate the symptoms of Parkinson's disease**
IRAS project ID:	**256914**
Protocol number:	**N/A**
REC reference:	**19/SC/0138**
Sponsor	**TdeltaS Ltd**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

Appendix 3 Participant information sheet sample

Participant information sheet for the 28-day study (chapter 5) is shown. There are similar sheets for the endurance exercise and ^{31}P-MRS studies.



TAS delta

NHS Oxford Health NHS Foundation Trust

UNIVERSITY OF OXFORD

PARTICIPANT INFORMATION SHEET

Can a ketone drink (ΔG®) alleviate the symptoms of Parkinson's disease?

You (and possibly a care partner) are being invited to take part in a research study as you have expressed an interest in doing so. Before you decide to participate, it is very important for you to understand why the research is being done and what it will involve. You can decline to participate in this study at any point.

Please take time to read the following information carefully.

This participant information leaflet is split into two parts:

Part 1

Describes the purpose of this study and what will happen to you if you decide to take part.

Part 2

Has more detailed information about how we will conduct the study.

Please make sure you ask us if there is anything that is not clear or if you would like more information.

Part 1

The purpose of the study and what will happen to you if you take part.

1. Why have I been invited?
You have been identified as an indivudal who may meet the eligability criteria for our study. We hope that you will consider participating in our study and, thereby, make a contribution to the advancement of Parkinson's research.

2. What is the purpose of the study?
To investigate the hypothesis ketone bodies may improve the symptoms of Parkinson's.

3. Do I have to take part?
No, it is up to you to decide whether to take part. If you do, you will be given this information sheet to keep and will be asked to sign a consent form. You are free to withdraw at any time without giving a reason. A decision to withdraw, or a decision not to take part, will not affect the standard of any medical care that you may need in the future.

4. What will happen to me if I take part?
Your participation in this study would involve fortnightly visits over the course of 2 months to the John Radcliffe Hospital. Visits may also occur at the Sherrington Building of the Department of Physiology, Anatomy and Genetics at Oxford University, or your own home. Each visit will likely last 1-2 hours but can last longer under special circumstances.

During the first visit we will ask you some questions to check whether you are eligible for the study and, if you agree to participate, we will ask you to sign a consent form and randomly assign you to either the experimental/ketone group or the placebo control group (see more below).

The first 2 weeks of the study will constitute a baseline period. The following 4 weeks constitute the actual intervention. During the intervention, you will be asked to ingest your respective drink (ketone or control) 4 times daily. You will also be asked to come in for a follow-up assessment 2 weeks after the conclusion of the intervention.

At each fortnightly visit (excluding the enrollment visit) you will undergo a series of noninvasive motor and nonmotor tests and be asked to provide blood samples. The motor and nonmotor tests include minimally burdensome easy-to-perform tasks such as standing, walking, smelling, reading, naming colors, and filling out brief questionnaires. We will take blood samples through a small needle inserted into one of your arms to measure levels of various metabolites.

5. What else do I have to do?
Apart from the tests described above, you will be asked to wear a small no-maintenance continuous activity monitor on your lower back for 7 days before and 7 days during the intervention. You will also be asked to use a 7-minute smartphone app 4 times every day throughout the trial. To assess compliance, we will ask you to record each time you consume a study drink in a diary. We will also call

you once per week at random to ask you when you last consumed a study drink, to ask you about your energy level, and request that you measure you ketones by urine stick. We will provide a smartphone preloaded with the app and a ketone monitor and show you how to use both.

6. If my partner/carer is involved what will s/he have to do?

During the visits that occur at the beginning and end the of the intervention period (second and fourth visits) your partner/carer will be asked to fill out two questionnaires. (1) The first questionnaire is used to assess whether you may have a sleep disorder. You and your partner/carer will fill this out together as s/he may be able to provide information about which you are unaware, such as if you occasionally act out your dreams. This information will be used to categorize your data into a subgroup for the purposes of some of our analyses. (2) The second questionnaire is a quality of life questionnaire your partner/carer will fill out at the same time you fill out a similar quality of life questionnaire. We ask this of partners/carers because we recognize that the impact of Parkinson's disease can extend beyond the individual.

7. What is the drink that is being tested?

The ketone ester is called ΔG® and is transformed into ketone bodies after being digested. These substances are not naturally found in the human diet but are produced by the liver during carbohydrate starvation as an alternative energy source for the brain.

ΔG® is produced by the parent company TΔS, a spin-out company from the University of Oxford's Department of Physiology, Anatomy and Genetics. ΔG® was first invented by Oxford's own Professor Kieran Clarke using a $10 million grant she received from the United States military to create a product that would enhance soldiers' physical and cognitive performance.

8. What are the potential side effects drinking the ketone ester ΔG®?

ΔG® is generally well tolerated. You may experience none, some, or all mild episodes of the symptoms listed below:

- Diarrhoea
- Abdominal distension
- Nausea
- Headache
- Dizziness

Published studies, in rodents and healthy human subjects, support ΔG®'s safety and tolerability for longer-term use. In collaborations with UK Sport, numerous studies have been performed on ~250 athletes to determine the effects of single drinks on physical endurance and cellular metabolism without problems. The HVMN ΔG® drink has FDA approval and is commercially available in the United States as a sports supplement.

9. What are the other possible disadvantages and risks of taking part?

Some participants find venepuncture painful or have difficult to access veins. To minimise these problems, all venepunctures will be performed by NHS-certified personnel.

10. What are the possible benefits of taking part?
You may experience improvements in motor symptoms, cognitive function, sleep, smell, and mood. It is also possible that you will experience no noticeable benefits.

11. Will ΔG® be available after the study ends?
If would like to purchase ΔG® for yourself after the study is complete, it is currently commercially available as a sports supplement from the biotech company HVMN. The following is a link to the company's website: https://hvmn.com/ketone. It is currently very expensive, although the price is expected to come down as the synthesis process streamlined and scaled up.

12. What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. Contact information is provided below.

13. Will my taking part in this study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. More details about this are included in Part 2.

14. Will I receive reimbursement for taking part in this research?
Yes. Participants who complete the study will receive £150 and reasonable travel expenses.

15. Who do I contact if I have problems?
If you wish to complain about any aspect of the way in which you have been approached or treated during this study, you should contact the investigators directly at 07444 054375 or nicholas.norwitz@dpag.ox.ac.uk. You may also contact the University of Oxford Clinical Trials and Research Governance (CTRG) office at 01865 572224 or the head of CTRG at ctrg@admin.ox.ac.uk. Alternatively, you can contact Chief Investigator, Professor Michele Hu, at michele.hu@ndcn.ox.ac.uk.

The Patient Advisory Liaison Service (PALS) is a confidential NHS service that can provide you with support for any complaints or queries you may have regarding the care you receive as an NHS patient. PALS is unable to provide information about this research study.

If you wish to contact the PALS team, please contact:
PALS Office
Churchill Hospital
Old Road, Headington
Oxford OX3 7LE
Tel: 01865 235855
Email: PALSCH@ouh.nhs.uk

TΔS® Ltd, as Sponsor, has appropriate insurance in place in the unlikely event that you suffer any harm as a direct consequence of your participation in this study. NHS indemnity operates in respect of the clinical treatment which is provided.

Part 2

More detailed information about how we will conduct of the study

1. Who is organising, sponsoring, and funding the research?
The Department of Physiology, Anatomy and Genetics (DPAG) of the University of Oxford is organizing the research, TΔS® Ltd is sponsoring the research, and a private benefactor is funding the research through the sponsor via a designated donation.

2. What will happen if I don't want to carry on with the study?
You can withdraw from the study at any point.

3. Will my taking part in this study be kept confidential?
All the information is confidential. None of the information stored on computers will be identifiable with your name. We will replace your name, initials, and date of birth with a participant number to make sure you remain anonymous.

4. What will happen to the samples that I give?
The samples you provide will be anonymised using a study specific de-identification code. They may be transferred to the University of Oxford for analysis or analysis may occur at the John Radcliffe Hospital's laboratories. Before analysis is complete, your data will remain de-identified and secure in a locked drawer, when it is not being actively analysed by a member of the research team. After the samples have been analysed, they will be immediately destroyed.

5. What if my samples reveal an abnormal finding?
All clinically relevant information will be relayed to your General Practitioner.

6. Will any genetic tests be done?
No genetic tests will be performed as part of this study.

7. Who is responsible for and what will happened with my information?
TΔS Ltd. is the sponsor for this study based in the United Kingdom. We will be using information from you and/or your medical records in order to undertake this study and will act as data as the data controller for this study. This means that we are responsible for looking after your information and using it properly. TΔS will keep your information for 5 years after the study has finished. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we

will keep the data we have already obtained. To safeguard your rights, we will use the minimum possible personally identifiable information. The John Radcliffe Hospital will collect information from you and/or your medical records for this research study in accordance with our instructions.

The John Radcliffe Hospital will use your name, NHS, number, and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from TΔS, the University of Oxford, and regulatory organisations, may look at your medical and research records to check the accuracy of the research study. The John Radcliffe Hospital will pass these details to TΔS along with the information collected from you and/or your medical records. The only people at TΔS who will have access to information that identifies you will be people who need to contact you if any health issues arise or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number, or contact details.

The John Radcliffe Hospital will keep identifiable information about you from this study for up to 5 years after the study has finished.

8. What will happen to the results of the research study?
At the end of the study, the results will be presented at regional, national, and international meetings and published in medical journals. All published results and information will be anonymised.

9. Who has reviewed the study?
This study has been given a favourable ethical opinion for conduct in the NHS by South Central – Oxford B Research Ethics Committee.

This study is part of an educational project that will contribute towards doctoral degrees in Physiology, Anatomy, and Genetics for DPhil (PhD) candidates Nicholas Norwitz and Dr. Adrian Soto.

If you would like to be part of this study, or would like more information, please contact Nicholas Norwitz.
Email: nicholas.norwitz@dpag.ox.ac.uk
Mobile number: 07444 054375

Appendix 4 Informed consent sample

Informed consent form for the 28-day study (chapter 5) is shown. There are similar consent forms for the endurance exercise and ^{31}P-MRS studies.

Department of Physiology, Anatomy and Genetics; Sherrington Road, OX1 3PT; Version 1.10, 11-03-20

TAS delta

NHS Oxford Health NHS Foundation Trust

UNIVERSITY OF OXFORD

PARTICIPANT CONSENT FORM

Study Full Title	A ketone drink (ΔG®) to alleviate the symptoms of Parkinson's IRAS identification number: 256914 ISRCTN64294760 Purpose of the study: To test the hypothesis that ingestion of a ketone ester supplement will improve the symptoms of Parkinson's
Researchers	PI: Prof Michele Hu Tel: 01865 231295 michele.hu@ndcn.ox.ac.uk Primary researcher: Nicholas Norwitz (DPhil student) Tel: 07444 054375 nicholas.norwitz@dpag.ox.ac.uk

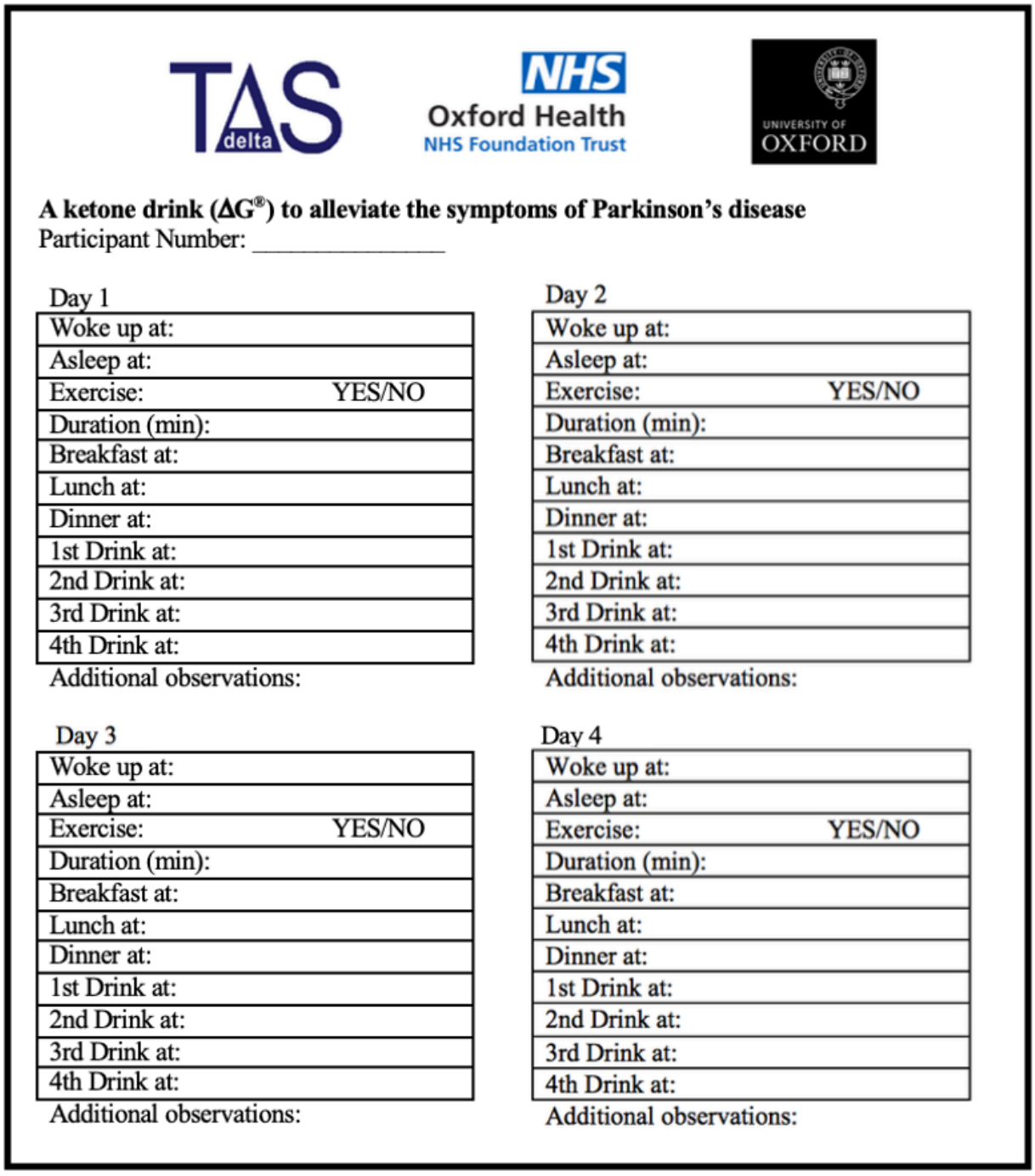
1. I confirm that I have read and understood the information sheet (version 1.14) for the above study. I have had time to consider the information, ask questions and have had these answered satisfactorily. _____
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without penalty or affecting my legal rights. _____
3. I understand that research data collected during the study may be looked at by designated individuals from the University of Oxford, regulatory authorities, or John Radcliffe Hospital, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data. _____
4. I agree for research data collected in this study to be given to researchers, including those working outside of the EU, to be used in other research studies. I give permission for data from this study to be used in publication. I understand that any data of me that leave the centre will be fully anonymised so that I cannot be identified. _____
5. I understand who will have access to personal data provided, how the data will be stored, and what will happen to the data at the end of the project. _____
6. I understand how to raise a concern or make a complaint. _____
7. I understand that the research team may contact my doctor. _____
8. I understand that this is a research project that is not useful for medical diagnoses. If a concern is raised about a possible abnormality during the project, I will only be informed if a doctor thinks it is medically important such that the finding has clear implications for my current or future health. _____
9. I agree to enroll in this study. _____
10. *Optional:* I agree to my contact details being kept after the study to allow the study team to contact me in the future to ask if I am interested in future related studies.

YES / NO

Name of participant	Signature	Date
Name of person taking consent	Signature	Date

Appendix 5 Drink diary

As one measure of compliance on the 28-day study, participants record when they consumed their study drinks.



A ketone drink (ΔG®) to alleviate the symptoms of Parkinson's disease

Participant Number: _____

Day 1

Woke up at:	
Asleep at:	
Exercise:	YES/NO
Duration (min):	
Breakfast at:	
Lunch at:	
Dinner at:	
1st Drink at:	
2nd Drink at:	
3rd Drink at:	
4th Drink at:	

Additional observations:

Day 2

Woke up at:	
Asleep at:	
Exercise:	YES/NO
Duration (min):	
Breakfast at:	
Lunch at:	
Dinner at:	
1st Drink at:	
2nd Drink at:	
3rd Drink at:	
4th Drink at:	

Additional observations:

Day 3

Woke up at:	
Asleep at:	
Exercise:	YES/NO
Duration (min):	
Breakfast at:	
Lunch at:	
Dinner at:	
1st Drink at:	
2nd Drink at:	
3rd Drink at:	
4th Drink at:	

Additional observations:

Day 4

Woke up at:	
Asleep at:	
Exercise:	YES/NO
Duration (min):	
Breakfast at:	
Lunch at:	
Dinner at:	
1st Drink at:	
2nd Drink at:	
3rd Drink at:	
4th Drink at:	

Additional observations:

Appendix 6 Consumer questionnaire

The questionnaire administered to participants of the 28-day study to evaluate their subjective experiences with the study drink.

1. **How did you find the taste of the study drink?** *(Please, circle one number.)*

   Intolerable – 0 1 2 3 4 5 6 7 8 9 10 – Very pleasant

2. **Did you find that it became easier or harder to ingest the study drink over the course of the month?** *(Please, circle one response.)*

   Easier Harder No change

3. **Please use three words that best describe your experience of the study drink.**

   _____

4. **Please rate your energy level as a result of consuming the study drink?**

   Much lower – 0 1 2 3 4 5 6 7 8 9 10 – Much higher

5. **Did you experience any benefits associated with the study drink?** *(Feel free to respond in any way you wish. Continue overleaf if necessary.)*

   _____

6. **Did you experience any problems associated with the study drink?** *(Feel free to respond in any way you wish. Continue overleaf if necessary.)*

   _____

7. **Do you think the study drink had any effect on how your Parkinson's medication usually work?** *(Feel free to respond in any way you wish. Continue overleaf if necessary.)*

   _____

8. **If this study drink were available to buy in a shop, how likely would you be to recommend it to other people with Parkinson's?** *(Please, circle one number.)*

   Not at all – 0 1 2 3 4 5 6 7 8 9 10 – Very likely indeed

Appendix 7 Ketogenic diet for mental illness video abstract

A video abstract that summarizes, in 4:28 minutes, the contents of the below manuscript can be found on the publisher's website at the following link: http://links.lww.com/COE/A16.

CURRENT OPINION

Ketogenic diet as a metabolic treatment for mental illness

Nicholas G. Norwitz[a], *Shebani Sethi Dalai*[b], *and Christopher M. Palmer*[c]

Purpose of review
Ketogenic diets, which have been used to treat drug-refractory paediatric epilepsy for over 100 years, are becoming increasingly popular for the treatment of other neurological conditions, including mental illnesses. We aim to explain how ketogenic diets can improve mental illness biopathology and review the recent clinical literature.

Recent findings
Psychiatric conditions, such as schizophrenia, depression, bipolar disorder and binge eating disorder, are neurometabolic diseases that share several common mechanistic biopathologies. These include glucose hypometabolism, neurotransmitter imbalances, oxidative stress and inflammation. There is strong evidence that ketogenic diets can address these four fundamental diseases, and now complementary clinical evidence that ketogenic diets can improve the patients' symptoms.

Summary
It is important that researchers and clinicians are made aware of the trajectory of the evidence for the implementation of ketogenic diets in mental illnesses, as such a metabolic intervention provides not only a novel form of symptomatic treatment, but one that may be able to directly address the underlying disease mechanisms and, in so doing, also treat burdensome comorbidities (see Video, Supplementary Digital Content 1, http://links.lww.com/COE/A16, which summarizes the contents of this review).

Keywords
inflammation, ketogenic diet, mental Illness, oxidative stress

Appendix 8 List of achievements and projects

A concise summary of the projects related to ketogenics, nutrition, and neurodegenerative and neurological diseases that are completed or ongoing.

PUBLICATIONS AND PRESENTATIONS

Peer-Reviewed Publications

(1) **Norwitz NG,** Hu MT, Clarke K. The mechanisms by which the ketone body D-β-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease. *Frontiers in Nutrition*. 2019

(2) **Norwitz NG,** Mota AS, Norwitz SG, Clarke K. Multi-loop model of Alzheimer disease: an integrated perspective on the Wnt/GSK3β, α-synuclein, and type 3 diabetes hypotheses. *Frontiers in Aging Neuroscience*. 2019 (Impact Factor: 4.5)

(3) Mota AS, **Norwitz NG**, Clarke K. Why a D-β-hydroxybutyrate monoester? *Biochemical Society Transactions*. 2020 (Impact Factor: 3.4)

(4) **Norwitz NG,** Loh V. A standard lipid panel is insufficient for a patient on a high-fat, low-carbohydrate ketogenic diet. *Frontiers in Medicine*. 2020 (Impact Factor: 3.4)

(5) **Norwitz NG**, Sethi S, Palmer C. Ketogenic diet as a metabolic treatment for mental illness. *Current Opinion in Endocrinology and Diabetes*. 2020 (Impact Factor: 3.5)

(6) **Norwitz NG**, Querfurth H. mTOR mysteries: nuances and questions about the mechanistic target of rapamycin in neurodegenerative disease. *Frontiers in Neuroscience*. 2020 (Impact Factor: 3.6)

(7) **Norwitz, NG**, Dearlove DJ, Lu M, Clarke K, Dawes H, Hu MT. A ketone ester drink enhances endurance exercise performance in Parkinson's disease. *Frontiers in Neuroscience*. 2020 (Impact Factor: 3.6)

(8) **Norwitz NG,** Naidoo U. Nutrition as a metabolic treatment for anxiety. *Frontiers in Psychiatry*. 2020 (Impact Factor: 3.5)

Book Chapters

(1) **Norwitz NG,** Jaramillo JG, Clarke K, Mota AS. Ketotherapeutics for neurodegenerative disorders. *International Review of Neurobiology*. 2020

Presentations

(1) **Norwitz NG.** An exogenous ketone ester for Parkinson's disease. Poster in Oxford, England. November 2019.

(2) **Norwitz NG.** Ketogenic diet to prevent neurodegenerative disease. Conference in Oxford, England. July 2019.

Editorial Activities

(1) Reviewer for *Nutrients* (4 manuscripts), *Physiology & Behavior* (1 manuscript), *Bioscience Reports* (1 manuscript), and *Nutrition Research* (1 manuscript)

EDUCATION AND COMMUNITY OUTREACH

Food as Medicine Co-Director

- Co-director and chief lecturer for Harvard Medical School's elective, *Food as Medicine: Mechanisms, Myths & Mysteries*.
- Chiefly responsible for developing course content and delivering regular lectures to the Harvard Medical School first year students.

Cookbook Author

- Lead author and coordinator of a science-based cookbook that includes biomedical background chapters, unpresented nutritional analyses (generated using the MUNCH application), and nutrition fun facts.
- Publisher: Fair Winds Press; manuscript complete; publication date: Spring 2021.

MUNCH Nutrition Tracking Application Developer

- Inventor and creator of My Ultimate Nutrition Calculator and Helper (MUNCH), a first-of-its-kind nutrition tracking application designed for researcher and clinical use.
- MUNCH allows the user to track any nutrient intake variable, including and especially those that cannot be tracked on other platforms; currently available as a web- and smartphone-based version 1.

Restaurant Consultant and Event Organizer

- Consult for head chef at Oxford's QUOD restaurant, R. Kashid, to provide ketogenic menu options.
- Organize nutrition education themed dining events to bring together members of Oxford community.

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