

Paraquat and Parkinson's Disease
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A. Richard Krolewski, M.D., Ph.D.: Background and Qualifications

I, Richard C. Krolewski, M.D., Ph.D. am currently an Instructor in Neurology Harvard Medical School, an Associate Neurologist at Brigham and Women's Hospital, and member of the Movement Disorder Division in the Department of Neurology. I have a Bachelor of Science from Syracuse University (Syracuse, NY). My medical training and graduate doctoral work was completed in the MD/PhD program at Tufts University School of Medicine in Boston, MA in 2012. During my PhD training, I studied how neurons and other cells in the olfactory system of rodents are affected by exposure to an inhaled toxicant. This inhalational exposure results in death of these neurons. I used this system and the subsequent response of the olfactory cells as a way to learn about how neurons can be regenerated[1-5]. My graduate medical training continued in neurology residency and movement disorders fellowship at Brigham and Women's Hospital and Massachusetts General Hospital, both in Boston, MA. During residency and fellowship, I worked in Professor Lee Rubin's lab at Harvard University in the Harvard Stem Cell Institute. I developed methodologies to study the effects of pesticides on dopaminergic neurons, a critical group of neurons affected in Parkinson's Disease. This methodology allowed our group and collaborators to model gene environment interactions using induced-pluripotent stem cells (iPS cells) from Parkinson's disease patients. My work was pivotal in securing multiple large collaborative grants from the Department of Defense (2019-2022, 2023-2027) to study pesticides and gene-environment interactions in Parkinson's disease. Initial findings from this project were published in the journal *Nature Communications* in collaboration with Dr. Beate Ritz and colleagues at UCLA [6]. As of the writing of this report, our article has been accessed over 15,000 times and our article is in the 99th percentile of 405,161 tracked articles of a similar age in all journals according to Altmetric analytics. I have given multiple invited presentations on this topic: at the Parkinson's Study Group National meeting in Phoenix, Arizona in June 2022, at the Pittsburgh Institute for Neurodegenerative Diseases at the University of Pittsburgh in January of 2024, and at Neurology grand rounds at the University of California at Los Angeles in June of 2024. I have served as an expert reviewer internationally, including for the French National Agency for Research and ParkinsonNL. I have served as an *ad hoc* reviewer for multiple journals including NPJ Parkinson's Disease, Science Advances, Molecular Neurobiology, Cell Stem Cell and the Neurohospitalist. I was awarded a Neuroscience Research Training Scholarship from the American Academy of Neurology and currently hold a 5-year mentored clinician-scientist research award (K08 grant) from the National Institute of Environmental Health Sciences to continue this work. This project, which I lead in the lab of Dr. Vikram Khurana at Brigham and Women's Hospital, focuses on the effects of pesticides on different brain cell types derived from patients with Parkinson's Disease. With the second large collaborative grant from the Department of Defense, we have expanded the work to include an assessment of the cognitive effects of pesticide exposure and changes to the gut microbiome as a result of pesticide exposure in patients with and without Parkinson's disease dementia. I have also been involved in computational projects that attempt to use large clinical data sets to improve speed and diagnostic accuracy in Parkinson's disease [7]. My larger career goal is that

the data on gene-environment interactions I help to generate will improve our understanding of the effects of pesticides. In turn, this will ultimately help inform and alter public policy to reduce the use of pesticides that increase the risk of PD, thus lowering the incidence of this debilitating disease.

As a clinician scientist with an active clinical practice in addition to my research program, I specialize in patients with Parkinson's disease, tremor, and related disorders. I have had over a thousand outpatient visits with patients who have Parkinson's disease, atypical Parkinsonism, and tremor. I have made the diagnosis of Parkinson's disease in dozens to hundreds of patients in my clinical practice. I have completed the International Movement Disorders Society "MDS-UPDRS Training Program & Certificate Exercise" to help standardize my examination of Parkinson's disease patients according to an internationally accepted rating scale designed to improve clinical trial accuracy and reproducibility [8]. As a respected referral center, patients travel across the country and internationally to receive care from me and my colleagues for their Parkinson's disease and related movement disorders. As such, I have the privilege to care for an incredible breadth and complexity of Parkinson's disease patients and complications thereof. Many of my patients go on to require advanced neurosurgical therapies for their symptoms including deep brain stimulation and focused ultrasound. I am co-director of our Division's research conference series where we host clinicians and scientists from around the country and internationally to discuss advancements in the basic science underlying Parkinson's disease and ongoing clinical trials and therapeutic developments.

B. Methodology

I evaluated published and unpublished data from multiple sources in the preparation of this report. The primary methodology involved using the National Center for Biotechnology Information literature search engine "PubMed" (<https://pubmed.ncbi.nlm.nih.gov/>). In order to monitor for the newest published findings, I have also set a periodic PubMed notification such that every month I receive a list of any newly indexed PubMed citations containing the word "paraquat". PubMed search terms used to return the most relevant results included "Paraquat AND Parkinson's Disease"; "Paraquat AND neurodegeneration"; "paraquat AND neuronal death"; "pesticides AND Parkinson's Disease". In my literature search, I attempted to identify publications that spanned a breadth of methodologies and model systems. These included basic cell biology, *in vitro* work (using cells grown or maintained in a sterile environment in the lab, not in a living organism) with Parkinson's disease relevant cell types, *in vivo* work (living animals used for experiments) with various model organisms from fruit flies to rodents to primates, and epidemiology involving human subjects with or without Parkinson's disease and related disorders. This report is not intended as a comprehensive review of all literature relevant to Parkinson's disease and paraquat, but instead highlights landmark studies, studies that are in opposition to the landmark studies, and important conceptual advances in the field that inform my opinions about causality.

Additional search methods utilized but relied on less heavily included Google Scholar as well as utilizing references cited in textbooks and other publications by other experts in the field of

Parkinson's disease. This includes the text book "Principles and Practice of Movement Disorders" by Fahn, Jankovic and Hallett as well as the lay audience-oriented "Ending Parkinson's Disease" by Dorsey *et al* [9, 10].

When weighing the quality of data and strength of evidence for a basic science, *in vitro*, or animal model providing relevant data, I took into account the specific methodologies used in each study I reviewed, whether the work had been peer reviewed, the quality and reputation of the Journal publishing the work, and to what extent other groups have reproduced the data or offered data that is supportive of or consistent with the data presented in preceding work. I also consider potential conflicts of interest on behalf of researchers or entities sponsoring the research.

Editorials were also reviewed to aid in the identification of controversial data, findings, claims and issues in the field of paraquat, pesticides, and Parkinson's disease. These works often helped to identify oppositional viewpoints and offer useful counter arguments by which new data could be evaluated. Some of the work reviewed is also from "pre-print" servers (bioRxiv, medRxiv). These are manuscripts that have not yet been peer reviewed. These manuscripts are considered with additional scrutiny and reduced weight as they have not passed through a rigorous peer-review process.

In forming my opinion on the causal relationship between paraquat and Parkinson's disease, I applied criteria outlined by Hill in 1965 [11]. These criteria include: strength of association, consistency of observed association, specificity of the association, temporality, biological gradient, plausibility, coherence, experimental evidence, and analogy. During my discussion of the data relevant to assessment of causality as it relates to paraquat exposure and development of Parkinson's disease, I will refer back to these nine Bradford Hill criteria with considerations of the more advanced experimental, genetic, and bioinformatic methods available [12].

C. Parkinson's disease and its causes

Parkinson's disease (PD) is a complex, multi-factorial neurodegenerative disease. The hallmark clinical findings include rigidity (stiffness), bradykinesia (slowness of movement), and rest tremor (shaking) [13, 14]. Underlying many of these disabling symptoms is a loss of a specific type of neuron in the brain, dopaminergic neurons—named as such because they produce a chemical called dopamine that is involved in communication between adjacent neurons [14, 15]. As a critical number of the dopamine producing cells in a region called the substantia nigra of the midbrain become diseased and die, the motor symptoms of PD arise. In most cases, aggregates of the protein alpha synuclein in Lewy bodies in specific midbrain dopaminergic (mDA) neurons are seen in the brains of PD patients[16] . Given the strong correlation of dopamine neuron loss with clinical symptoms and the appearance of abnormal alpha synuclein aggregates in the brains of affected patients, substantial resources have been directed towards understanding what causes these processes to occur in patients [15, 17]. The abnormal aggregation and neuronal cell loss observed in PD occurs in neuroanatomical patterns that have

led researchers to evaluate two primary methods of disease propagation: 1) a pathway initiated in the gut/intestine where abnormal protein aggregates containing alpha synuclein are transported from the peripheral gut up into the brain along the vagus nerve that connects the brainstem to the intestines; and 2) a pathway originating in the olfactory system at the back of the nose, which then carries abnormal protein aggregates directly into the central nervous system and basal ganglia of the brain for subsequent propagation and spreading. Human autopsy data and epidemiologic study of patients who have had the vagus nerve cut for treatment of unrelated conditions along with carefully designed *in vivo* mouse model experiments have provided very strong evidence for the importance of these pathways from the gut and the nose in Parkinson's disease[18-22].

Environmental and genetic factors have been identified as causes of PD and risk of PD increases as patients age [23]. While only a small percentage of Parkinson's disease diagnoses can be attributed solely to genetic causes, the patients and families who carry genetic mutations which invariably cause PD have provided important insights into the types of cellular processes that when disrupted, can lead to death of dopaminergic neurons and therefore PD. Clues offered by these dramatic cases of familial PD have helped to identify environmental causes of PD by pointing towards nodes of vulnerability in function and survival of dopaminergic neurons [24-29]. The natural history and timing of familial PD also provides a useful framework for understanding the Bradford Hill criteria of **temporality** of exposure influencing development of Parkinson's disease. There exist well-described dominantly inherited forms of Parkinson's disease such that inheriting one copy of the abnormal gene is sufficient for development of the disease in the majority of patients [28, 30]. Even in these families where the inherited mutation results in development of Parkinson's disease, this disease does not manifest for decades. One report cites the age of onset as 46 years +/-13 years[30]. This demonstrates a substantial lag time between presence of the causative insult in the brain (the mutated, abnormal protein) and the eventual development of the disease. In these patients, the abnormal form of the protein alpha synuclein is being made in neurons prior to birth and remains present in these neurons throughout life. The lag time seen with this type of genetic "exposure" suggests that an environmental exposure which will eventually cause Parkinson's disease may require decades of lag time to manifest as disease. Not all exposures behave in this manner. The most notable exception is exposure to the synthetic heroin contaminant MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [31]. A toxic metabolite, or byproduct of this compound is generated during contact with supporting cells of the brain, astrocytes. This toxic metabolic, MPP+ is then rapidly taken up by and concentrated in dopaminergic neurons. In these neurons it induces a cascade of mitochondrial damage, production of free radicals and reactive oxygen species which cause a rapid and dramatic death of dopaminergic neurons resulting in parkinsonism for these patients[31]. This compound has proven extremely useful for modeling the motor symptoms of Parkinson's disease in animal experiments, but it effectively takes a shortcut to the step of dopamine neuron death and dysfunction rather than reflecting the more prolonged progression of disease seen in genetic models or other toxicant models (such as the rotenone model) of Parkinson's disease [32].

Cell Biology of Dopaminergic Neurons: Patients with Parkinson's disease demonstrate a striking loss of a specific population of dopaminergic neurons in the deep structures of the brain (substantia nigra of the midbrain). These neurons participate in a complex circuit that regulates movement, also called motor function[33]. Structural examination of these neurons indicate they have extensive processes, and therefore extensive points of communication with the brain structures they extend into and receive information from multiple inputs. Functional examination of these neurons in living animals and following removal from experimental animals has revealed that these neurons have a relatively unique "pacemaker potential" such that they have a regular pattern of activity and signal generation (called action potentials) even when the organism is not actively moving[34]. The frequency of activity is altered based on inputs and voluntary movement by the organism which in turn influences volitional movement or lack of movement. This requires that the dopaminergic neurons have an ongoing and consistent source of energy to produce these signals. Continued production of signals from dopaminergic neurons also requires ongoing production, use, transport, and recycling of dopamine—the chemical released by dopaminergic neurons to transmit their signals to other neurons in the brain. These functions place a number of demands on the dopaminergic neurons: it requires that they are energetically active and it requires that they can supply and deliver dopamine to the end of the cells (so called nerve terminals or synapses) [35]. A substantial amount of the energy produced by neurons (and cells in general) is generated in a compartment of the cell (organelle) called mitochondria. Cells performing more "work"—in the form of neuronal signals, movement of cellular structures, or contraction require large amounts of active and healthy mitochondria [36]. For example, muscle cells which actively contract to produce movement, have numerous large mitochondria to produce the fuel needed by the cell to do the work of contracting. Similarly, neurons which need to send frequent or rapid signals to other parts of the brain require consistent and active generation of energy by mitochondria. Some of the first known gene mutations identified as causes for familial (inherited) Parkinson's disease are involved in the proper removal of dysfunctional or damaged mitochondria from the cells which provides evidence for mitochondrial dysfunction as an important consideration in Parkinson's disease [37]. When mitochondrial dysfunction occurs, the amount of oxidative stress cells are subjected to is increased. Oxidative stress in the neuron has been identified as a putative convergence point of stress, dysfunction, and cellular injury to dopamine neurons[29, 38]. Dopamine neurons, by virtue of the fact that they must produce dopamine (rather than other neurotransmitters such as acetylcholine or serotonin) are subjected to additional oxidative stress by the chemical structure of dopamine itself and this excess oxidation of dopamine and its byproducts has been linked to multiple different mutations that cause or contribute to Parkinson's disease[38, 39].

Environmental factors: The characteristics of dopamine neurons described above are the endogenous factors that make them vulnerable. Exogenous factors have also been found to play an important role in PD risk and dopamine neuron vulnerability. Specifically, environmental exposures to chemicals, such as pesticides, that can either reach dopaminergic neurons directly in the brain or influence the severity of the various stressors listed above are expected to cause Parkinson's disease. In the same way that genetic mutations which cause mitochondrial dysfunction, increased oxidative stress, or dysregulated neuronal activity can

cause Parkinson's disease, environmental exposures that cause mitochondrial dysfunction, increased oxidative stress or dysregulated neuronal activity can also cause Parkinson's disease[40]. Multiple examples are notable in this regard and address the Bradford Hill criteria of **analogy**. For example, rotenone is a naturally occurring pesticide that disrupts the function of mitochondria. It has been linked to Parkinson's disease through epidemiologic exposure data[41]. When dopamine neurons are exposed to rotenone *in vitro* or when animals are exposed to rotenone *in vivo*, the dopamine neurons are sensitive and the animal develops deficits analogous to Parkinson's disease [32, 42, 43]. Trichloroethylene (TCE), an industrial solvent is another example[44]. Marine corps veterans exposed to this solvent at Camp Lejeune had a 70% higher risk of developing PD, corroborating other smaller epidemiologic studies. Basic science data indicates that exposure to TCE causes loss of dopaminergic neurons, mitochondrial dysfunction, and aggregation of the Parkinson's disease protein, alpha synuclein [45]. Paraquat, an herbicide, also fits into this conceptual framework as well and will be reviewed in detail below.

D. Paraquat exposure and Parkinson's Disease

Epidemiologic studies of Parkinson's disease patients across geographies and ethnicities have linked Parkinson's disease risk to pesticide exposure. A small study conducted in the midwestern United States comparing rural and urban subjects with and without Parkinson's disease pointed to pesticide use as a risk factor for PD, conferring a more than 3-fold risk of PD[46]. Data from Costa Rica indicated a higher risk of symptoms consistent with Parkinson's disease (slowed finger tapping or tremor at rest) in people with occupational exposure to pesticides in a case control study (again, approximately 2.5 fold) [47]. In Taiwan, a case-control study indicated that the use of herbicides/pesticides was associated with increased risk of PD and increasing cumulative lifetime doses conferred increased risk of PD[48]. This study is particularly compelling because it supports a dose-response with pesticide exposure- namely that lower amounts of pesticide exposure increase risk but higher amounts of pesticide exposure increase risk more than the lower amount of exposure.

As data emerged supporting an increased risk of Parkinson's disease with exposure to pesticides, the obvious next step for researchers to take was to obtain more granular data on which pesticides might be particularly problematic or confer the most risk. The much larger Farming and Movement Evaluation study of patients and controls in northern California and Iowa (110 PD cases, 358 controls) indicated that exposure to the herbicide paraquat conferred a 2.5 fold increased risk of PD[41]. The study by Liou *et al* in Taiwan mentioned above also identified paraquat as a specific risk factor for PD with a dose-response relationship [48]. Specifically, subjects with 1-19 years of paraquat exposure had an odds ratio of 1.19 (not statistically significant) for the association of PD and paraquat exposure, while subjects with >20 years of exposure had an odds ratio of 6.41, which was statistically significant [48]. This principle of dose response is an example of **biological gradient** and is one of the Bradford Hill criteria for causation mentioned above in section C [11]. Additional data from the central valley of California provides further evidence for paraquat exposure as a cause of Parkinson's Disease by describing changes in disease risk as a function of exposure timing, amount, and additive

effects with other pesticides, such as maneb[49, 50]. I have given substantial weight to the results of the studies by Liou *et al*, Tanner *et al*, and the Ritz group in part because the patients in these studies were individually examined by movement disorders neurologists involved in the study instead of relying on chart review. This increases the likelihood that cases and controls were correctly classified given the improved accuracy of diagnosis when examined by a movement disorders specialist [51]. Additional weight is given in particular to the Tanner *et al* findings as the primary data was made available to a consulting group (Exponent) for a sensitivity analysis (SYNG-PQ-36932783). This independent repeat analysis was funded by Syngenta and “exhibited a degree of robustness” such that the data were consistent and robust even with “some artificially introduced exposure classification errors” with only a slight decrease in the odds ratio in this analysis. The Tanner study also provides data in the form of an easily interpretable, quantifiable, and applicable criteria to categorize an amount of paraquat exposure that differentiates the control group from the Parkinson’s disease group. In this study, the median days of paraquat exposure in controls was 8 days as described in supplemental table 3 [41]. Men exposed to paraquat at greater than the median duration of 8 days had an odds ratio of 3.6 (95% confidence interval 1.6-8.1) for association with Parkinson’s disease. I therefore consider this value as the minimum cumulative days of exposure at which point the risk of Parkinson’s disease is increased. This criterion is also useful for evaluation of different studies and exposure assessment because in a variety of studies the described exposure classifications are imperfect as there is not detailed information about use of personal protective equipment, dermal vs. inhalational exposure, and duration (i.e. seconds, minutes, hours) of exposure for each exposure event.

The breadth of geography, ethnicities, and methodologies used in these studies increases confidence in this result because this breadth reduces confounding based on study technique, genetics of a specific population or region, or unanticipated modifiers that could vary by country or region. Replication of the association across different studies also satisfies an additional Bradford Hill criterion of **consistency**[11]. A meta-analysis examining paraquat and Parkinson’s disease reported a “significant increase in risk of PD among subjects with a history of paraquat exposure. The overall OR was 1.64 (95% CI 1.27-2.13)”. The authors were careful to exclude studies that had overlapping or duplicated patient populations which could over-represent favorable studies. The authors appropriately pointed out that many studies did not assess levels and duration of paraquat exposure, and gave more weight to those that did. A discussion of the specific odds ratios for the association between paraquat and Parkinson’s disease in the above studies also allows for an evaluation of the Bradford Hill criterion of **strength of association**. The magnitude of the odds ratio in each of these studies (3.22 for Liou *et al*, 2.5 for Tanner *et al*, and 2.15 for Paul *et al*) is a reflection of the strength of association. This magnitude is comparable to the strength of association seen with broadly accepted genetic risk factors that “substantially” increase risk (certain APOE genes) for other neurodegenerative diseases such as Alzheimer’s disease[52]. In that case, an example odds ratio for a subset of patients in that study was 3.02. The odds ratios for paraquat and Parkinson’s disease do fall short of the strength of association for the canonical example of cigarette smoking and lung cancer—7.5 for former smokers and 23.6 for current smokers [53].

There are published studies that do not align with the results described above as it pertains to the Bradford Hill criteria of biological gradient, consistency and strength of association. Some of the studies are reviewed and discussed in a recent publication[54]. The conclusions provided in that review carry reduced weight in my analysis. Instead of performing a meta-analysis of the studies chosen for review—which is the gold standard when attempting to draw conclusions based on multiple published studies, the author instead provides a “review of reviews”. Furthermore, the review does not comment on the statistical likelihood of the multitude of studies which would all have to be making a Type I statistical error (supporting an association of paraquat exposure with PD risk if in reality there was no such association) to discount the results in aggregate. Multiple studies that failed to detect an association between Parkinson’s disease and paraquat exposure were also given reduced weight in my analysis because many did not utilize direct examinations by movement disorder neurologist to confirm the diagnosis of Parkinson’s disease. These studies instead relied on review of chart documentation, death certificate data, or non-expert clinical assessment, therefore increasing the likelihood that subjects were being misclassified [55-57]. The publication by Shrestha and colleagues reports a hazard ratio for ever/never use of paraquat that is non-significant and crosses the null value of 1 (1.09, range 0.84-1.41) [58]. While this analysis yielded a non-significant finding for paraquat, I consider this with reduced weight due to a few factors. The classification of ever/never as an exposure increases the likelihood of exposure misclassification and will increase variability. For example, a subject who applied paraquat once would be considered in the same exposure group as a subject who applied paraquat one hundred times. Data from other well-designed studies have provided evidence for a dose response and perhaps even a threshold for number of exposures in PD cases vs controls [41]. In addition, the Shrestha study did a subgroup analysis of subjects with history of head trauma, which is an independent risk factor for development of PD [23]. In these subjects, there was a significant association of both head trauma and paraquat exposure with PD (hazard ratio of 3.2)—as would be predicted for independent exposures that have additive or synergistic effects on the disease process. This allows for a stronger signal in the epidemiologic data. Additionally, in a follow up publication using data from the same large Agricultural health study, Yuan, Shrestha and colleagues identified paraquat as significantly associated with dream re-enactment behaviors with an odds ratio of 3.48 [59]. This is notable because dream re-enactment behaviors or REM sleep behavior disorder is a distinct clinical diagnosis that is also well-defined prodromal symptom of PD. Patients who exhibit dream re-enactment behaviors are at substantially higher risk of developing PD [60, 61]. This greatly strengthens the coherence argument for paraquat such that paraquat exposure is significantly associated with a completely independent diagnosis that is a known risk factor for PD. The study by Tomenson and Campbell is also in contradiction to the studies linking paraquat and PD [56]. This study reported on employees of plants that manufactured paraquat and analyzed death certificate data to draw conclusions about the association between paraquat exposure and PD. I have regarded this study with reduced weight because one of the authors is a Syngenta employee with a clear conflict of interest in the study outcome. Additionally, using death certificate data to identify cases of Parkinson’s disease is very likely to misclassify many subjects. Death certificate data is often inaccurate in Parkinson’s disease and related disorders. Many of the patients with PD will have suffered morbidity and disability from Parkinson’s disease, but died from other common causes of death in that age

group (i.e. cardiovascular disease, cancer, pneumonia) [62, 63]. A classification issue leads me to also discount the findings in a follow up study of acute paraquat poisoning [64]. The authors evaluated for parkinsonism, rather than Parkinson's disease. Parkinsonism is an umbrella term referring to findings seen in Parkinson's disease, but also including multiple other causative factors (for example: cerebrovascular disease and stroke, other related neurodegenerative disease, medications). Inclusion of these causes would result in inappropriate classification of the patients. The authors describe age characteristics of their population—with median of long-term survivors being 22 years old (mean 26 +/- 17 years) indicating that most of the data is from patients that are substantially younger than the age at which symptoms of Parkinson's disease manifest. A study by Van der Mark and colleagues is also in opposition to the principle of biological gradient or dose response [65]. This study describes an odds ratio of 1.27 (0.68-2.35) for the low paraquat exposure group and 1.03 (0.54-1.95) for the higher exposure group. These non-significant odds ratios argue against a biological gradient. This study enrolled patients with a later disease onset (67 years old) than other studies of paraquat (mean age in Tanner 61 years old, mean age in Liou is 58 years old) and descriptive studies of Parkinson's disease (61.6 years old) [66]. The older population in the Van der Mark may represent a bias in selection of patients and is therefore regarded with less significance.

Evidence from experimental systems: animal models and cell culture systems:

Due to the obvious practical and ethical constraints of performing experiments in humans related to agents suspected of causing disease in humans, the field turns to animal models of Parkinson's disease and the study of human cells and human neurons in a tissue culture experimental system to better understand the underlying biology of how pesticides like paraquat are likely to cause Parkinson's disease. While no animal model perfectly replicates the symptoms and pathology of Parkinson's disease in humans, there is substantial insight gained from experiments in these models. The following experiments address the Bradford Hill criteria of **experimental evidence**. When paraquat is given to primates, the function of dopaminergic neurons is altered, less dopamine is released in the striatum [67], and the protein alpha synuclein, a critical player in PD pathogenesis, is increased in brain samples at 2, 4, and 8 weeks after paraquat dosing (SYNG-PQ-0260198). When paraquat is given to mice, they experience loss of dopaminergic neurons in the midbrain and alpha synuclein aggregation in dopamine neurons, both of which are hallmarks of PD in humans [68-70]. Notably, in two separate reports of unpublished results, research commissioned by the manufacturer of paraquat confirmed previously published findings that mice exposed to paraquat alone (SYNG-PQ-00116782-MarksXm7258) and a combination of paraquat and maneb (Exponent Defra report) have decreases in the number of dopamine neurons in the substantia nigra. Other researchers have pursued the relationship between paraquat and maneb (a manganese containing fungicide) highlighted by the epidemiology. One group found that intraabdominal injection of paraquat, but not maneb, in mice increased diseased/pathological forms of the Parkinson's protein alpha synuclein [71]. One of the first groups to use neurons made from PD patient-derived induced pluripotent stem cells exposed these neurons to paraquat alone, maneb alone, and the combination of paraquat and maneb[24]. This work showed that paraquat kills patient neurons *in vitro* and the combination of paraquat and maneb shows synergistic toxicity—suggesting that both agents sensitize the neurons in different/complementary ways—complementing

epidemiologic data. There are published studies in opposition to the above data. A large *in vivo* mouse study performed on mice from two different suppliers and housed in different facilities did not detect a statistically significant decrease in dopamine neurons in the substantia nigra of paraquat treated mice seven days after treatment[72]. The authors obtained paraquat from two sources (Syngenta and Sigma) and attempted to replicate aspects of previously published studies by other groups—including timing of paraquat administration and animal sacrifice for analysis. This study was performed with a relatively large number of mice and aspects of the treatment and analysis were conducted in a blinded fashion—which prevents certain individuals from knowing the treatment which reduces bias. Despite these appropriate design methods and controls, the results of this study should be interpreted with caution given the conflict of interest that exists for the funding source (Syngenta) of the study and multiple authors deriving salary support from that funding source. Independent of that conflict of interest, the study highlights the complexity and variability of mouse models of Parkinson’s disease and leaves open the possibility that certain subtleties of handling or administration of paraquat (along with many other variables) could influence the experimental outcome. Studies by Breckenridge and colleagues delivered intraperitoneal doses or dietary exposure of paraquat to mice. These studies did not find consistent loss of dopaminergic neurons in the substantia nigra or alterations of dopamine and its metabolites in relevant regions of the brain [73, 74]. Again, there is concern about conflict of interest for some authors given that they were employed by Syngenta and the studies were funded by Syngenta. These publications utilized exposure methods that while commonly employed in research studies, were subsequently determined to result in much lower concentrations of paraquat in the central nervous system compared to inhalational exposure (see discussion of Exponent Defra data below). Subsequent research on mice has described an important difference between human cells and mouse cells with respect to calcium homeostasis and dopamine metabolism—two processes important in the cell biology of Parkinson’s disease[38]. These differences could underly variability seen in mouse models and contribute to increased sensitivity of humans to oxidizing stressors (including but not limited to paraquat). Additional reasons for inconsistent results in mouse studies in general in the neuroscience field has subsequently been attributed to differences in the microbial composition in the gut of mice that results from housing and husbandry in different mouse facilities [75]. Other experimental parameters have also been identified that can be challenging to control for [76, 77].

The discussion of paraquat and maneb co-exposure above raises the Bradford Hill criteria of **specificity**. Real world epidemiologic data is not expected to resolve this criteria adequately as all subjects exposed to paraquat in occupational settings are invariably exposed to multiple other pesticides and herbicides[6]. However, the extensive experimental data in animals (some of which is summarized above) and the extensive mechanistic experiments performed *in vitro* (some of which is summarized below) linking paraquat to the cell biology of Parkinson’s disease bolsters the case for specificity. In these experiments, the only toxicant introduced into the controlled experimental environment is paraquat. In these experiments there is specificity in that one exposure (paraquat) results in one “disease”—the experimental outcomes that are modeling Parkinson’s disease *in vitro* and *in vivo*.

The resultant abnormalities seen in the brains of model organisms or neurons *in vitro* have provided evidence for the mechanism by which paraquat causes neuronal cell death. Experiments and observations in multiple cell types and model organisms indicate that paraquat can contribute to cell death via multiple mechanisms that are likely initiated by free radical generation and oxidative stress by paraquat. Free radical generation and oxidative stress are problematic for cell survival for multiple reasons. Excessive oxidative stress alters or impairs the function of proteins doing the “work” of the cell by chemically modifying these proteins during aging and disease[78]. Elaborate systems exist within the cell to help neutralize/reduce oxidative stressors, but this system can be overwhelmed[79]. Excessive oxidation can also damage DNA—the critical set of blueprints that exist within each cell. DNA damage in turn triggers dysfunction and cell death[80]. Additional disruptions include lipid peroxidation, mitochondrial toxicity, and activation of inflammatory pathways[81]. Evidence of lipid peroxidation and oxidative stress have been reported in the substantia nigra of paraquat treated mice [68]. Treatment of cells *in vitro* with paraquat induces a well-defined cascade of programmed cell death called apoptosis and cells can be made less sensitive to paraquat by altering a specific component of this cell death pathway[82]. The experimental evidence describing free radical generation and oxidative stress caused by paraquat also address the criteria of **coherence** described by Bradford Hill. Ample experimental evidence links mitochondrial dysfunction and oxidative stress via multiple different methods unrelated to paraquat exposure (genetic mutations, energetic demands on the cells during normal function, exposure to various exogenous stressors or toxicants) to the changes seen in the brain of patients with Parkinson’s disease. It follows then that exposure to paraquat, a compound that causes mitochondrial dysfunction and oxidative stress is consistent with development of Parkinson’s disease. Separate from direct effects on mitochondrial dysfunction and oxidative stress, paraquat was shown to change the way that alpha synuclein (the Parkinson’s protein mentioned above) assembles into complex structures with itself, a process call oligomerization. Treatment *in vitro* and in mouse models of Parkinson’s disease *in vivo* indicated that paraquat caused a shift in the balance towards more pathologic/toxic forms of alpha synuclein rather than more physiologic or normally appearing complexes (tetramers) [83].

E. Plausibility of exposure methods resulting in brain pathology

Field studies have provided data that inhalational and dermal exposures exceed the acceptable operator exposure limit for sprayers and supervisors [84, 85]. Traditionally, there has been an appropriate focus on how well a pesticide (or chemical, or drug) can get into the central nervous system in order to come into contact with the vulnerable cell type and exert a direct effect on these cells, which in this case are the dopaminergic neurons of the substantia nigra—deep in the brain. The blood brain barrier is a tightly regulated impediment to substances in the bloodstream that regulates what can pass into the brain tissue and the fluid surrounding the brain (the cerebrospinal fluid). This barrier is made up of components and membrane processes from several cell types working in concert to regulate the passage of chemicals and proteins into the brain [86].

In the case of paraquat, multiple lines of evidence suggest that paraquat can penetrate the blood brain barrier. An internal document from the Chevron Chemical Company describes analysis of paraquat levels as part of an autopsy report. This was a patient who ingested 35mL of paraquat in a suicide attempt. The patient was found to have paraquat in their post-mortem brain tissue (date of death unclear from the document, but samples were described as received 18 days following ingestion). The amount detected in brain was two-thirds the level detected in the plasma (a type of blood sample) four days after ingestion and persisted in the brain after paraquat was no longer detectable in plasma (document ID CUSA-00168423). The organic cation transport system represents a plausible method by which paraquat can enter the human brain, as summarized in an internal memo by Dr. Lock (SYNG-PQ-20758518). The transporter Oct2 (a type of organic cation transporter) has been shown to facilitate the uptake of paraquat into human kidney cells and this transporter was also found to be expressed in the human brain [87, 88]. Evidence in mice finds paraquat in brain tissue following treatment with paraquat [89] and suggests that it utilizes a specific amino acid transporter to facilitate its transport across this barrier [87, 90]. In an internal study performed at Syngenta ("Gledhill, 2008"; document ID: SYNG-PQT-ATR-15302324), paraquat is described as being "only slowly eliminated from the brain of this animal" with a half-life of about 21 days, which was also corroborated by peer reviewed literature [91]. That internal review document also describes an internal study done in rats with radiolabeled paraquat indicating a half-life of about 28 days. Syngenta also analyzed squirrel monkey brains from experiments done by Dr. DiMonte and colleagues (Parkinson's Institute), who had dosed the monkeys with paraquat (SYNG-PQ-00044965; and email January 8th, 2011). This analysis found evidence of paraquat in the cortex of monkey brains two weeks after the exposure to paraquat finished and as long as eight weeks after the exposure finished. Similar levels, which were not statistically different from one another, per the document, were found at both these time points ("data from DiMonte.xls"). This suggests persistence of the herbicide and little if any reduction in the amount present in the cortex of the brain over that time interval. It is important to note that there is published data in primates from a few years prior to this communication using a sensitive detection system that describes "minimal uptake" of radiolabeled paraquat in rhesus macaques. This data argues against substantial blood brain barrier transport of paraquat in that model system [92] and these studies could be cited as reducing the coherence of the association between paraquat and Parkinson's disease. Interestingly, even if the negative data from primates proves reproducible or more representative of the process in humans, more recent research substantially diminishes the value of arguments contesting blood brain barrier penetration of disease-causing insults in Parkinson's disease. These data suggest that peripheral exposure of a disease-causing stimulus rather than direct exposure to the brain is sufficient to initiate Parkinson's Disease. The rationale for this is as follows: human pathological data, referenced above supports progression of Parkinson's disease from the lower brain stem (dorsal motor nucleus of the vagus) upward to other parts of the brain and from the olfactory system (olfactory bulb) [93]. This pathologic finding combined with epidemiologic data of patients post vagotomy (resection of the vagus nerve to help treat peptic ulcer disease—a condition unrelated to Parkinson's disease) and mouse experiments where the vagus nerve was intentionally severed, have provided compelling evidence that events with potential to initiate Parkinson's disease can happen in the intestine and then track upwards to the brain along the vagus nerve [94, 95]. By tracking up the

vagus nerve, the diseased propagating proteins enter the brain stem via this pathway rather than having to penetrate the blood brain barrier [95, 96]. In parallel to this process, or as a completely independent potential pathway, alpha synuclein pathology is found in the olfactory bulb of PD patients and the olfactory bulb has connections to the substantia nigra and locus coeruleus—two structures affected in Parkinson’s disease. Although not yet studied in the same mechanistic and anatomic detail as the vagus nerve pathway, this highlights another potential pathway for inhaled toxicants and pesticides to contribute to and cause Parkinson’s Disease[95]. Inhalational studies of paraquat exposure in rodents has demonstrated the presence of paraquat in multiple brain regions where there is neuronal loss and dysfunction in Parkinson’s disease—specifically the striatum, midbrain, brainstem, and cerebellum [97]. The olfactory mucosa and olfactory nerves are likely portals of entry after paraquat inhalation which allows the toxicant to evade typical blood brain barrier defenses and distribute into the multiple brain regions described above. This is based on the highest levels of paraquat being found in the olfactory bulb, which is part of the brain, following inhalation experiments in mice [97]. The olfactory bulb is the target of the olfactory nerve and the olfactory receptor neurons responsible for detecting odorants via contact with the outside environment of the nasal cavity. The Exponent defra report (project code PS2608) also provides evidence for rapid absorption after intranasal exposure of mice to radiolabeled paraquat that is more efficient than other methods of exposure tested. The percentage of the dose reaching the brain after intranasal exposure was nearly 20 times that of intraperitoneal exposure (0.526% for intranasal vs 0.027% for intraperitoneal) and nearly 50 times that of dermal exposure (0.011%). The authors appropriately note that “intranasal dosing in the PBPK model portion of this study indicated a fast and extensive circulation of paraquat in the brain tissue.” They also comment that “this preliminary observation has implications for exposure of humans to any neuroactive or neurotoxic substances that deposit in the nasal cavity during inhalation, including but not limited to pesticides.” If we allow for the possibility that applicators of paraquat consistently wear respirators that reduce or eliminate inhalational exposure to paraquat, internal data reveals that dermal absorption remains a concern as a possible method of exposure even in the scenario that personal protective equipment is worn (SY3B9E~1, Report No: CTL/P/1467, Paraquat: Penetration through protective clothing materials from a Malaysian ‘Gramoxone’ Formulation). This report states that Tyvek, corovin and unfinished cotton “displayed little resistance to paraquat penetration from either the concentrate or spray dilution.” Only coated Tyvek materials had resistance to paraquat penetration.

These findings provide a biologically plausible mechanism by which pesticides such as paraquat could cause Parkinson’s disease even if they are completely and perfectly excluded by the blood brain barrier. This would be accomplished by triggering formation of abnormal alpha synuclein folding outside the brain that is then relayed into the otherwise protected central nervous system. There is extensive data that this abnormal protein can then act as a “seed” which propagates the disease to adjacent neurons and adjacent brain structures[19, 20]. Paraquat exposure therefore meets the Bradford Hill criteria of **plausibility** in yet another way based on exposure methods independent of whether it penetrates the blood-brain barrier.

F. Regulatory considerations

Beyond the peer reviewed literature, multiple organizations have supported the conclusion that paraquat is a causal factor in Parkinson's disease. The National Health Surveillance Agency of Brazil in 2016 performed an extensive, data-driven review of the data for paraquat as it relates to Parkinson's disease (Anvisa/SYNG-PQ-06641275) and concluded "Considering the current scientific knowledge, it is concluded that Paraquat is mutagenic and is a risk factor in Parkinson's Disease, with evidence of causality between exposure and the disease". The Michael J Fox Foundation, which sponsors a rigorous, peer-reviewed research program with the goal of advancing treatments and care for PD patients has also taken on an advocacy role and lobbied Congress to pass legislation banning paraquat (<https://www.michaeljfox.org/news/earth-day-lets-ban-known-environmental-risk-parkinsons>). Paraquat has been banned in over 50 countries, including Brazil, China, and the European Union. One very notable exception to this is the United States. The Environmental Protection agency has not banned the use of paraquat. This is a policy I disagree with. In the summary review released in January 2024, the agency noted that approximately 90 submissions containing scientific studies were presented to the EPA by the Michael J Fox Foundation and EarthJustice but that the agency did not undertake a complete review of this updated information prior to issuing their assessment. I am hopeful that with a more complete review of these materials as well as consideration of more recent publications such as the data on paraquat from the PEG study by the Ritz group [50], the EPA will reconsider their assessment.

G. Summary

Multiple lines of epidemiologic evidence from different patient cohorts demonstrate significance for the association between paraquat exposure and Parkinson's disease. Exposure by agricultural workers who spray or are in proximity to paraquat has been a focus of the epidemiologic studies. Human autopsy data, field studies, and experimental animal studies identify both likely and plausible methods by which paraquat exposure results in central nervous system penetrance which then alters the biology of neurons that die in Parkinson's disease. Animal studies using paraquat, while imperfect models with imperfect applicability to humans with Parkinson's disease, result in cellular and biochemical changes consistent with processes that cause dysfunction or death in relevant populations of brain neurons. Experiments performed on various types of neurons generated *in vitro* that have been treated with paraquat provide robust mechanistic data that fits with the known vulnerabilities in the cell biology of PD patient neurons. **These data lead to my conclusion that paraquat exposure in humans is a cause of Parkinson's disease.** I hold all the opinions expressed in my report to a reasonable degree of medical and scientific certainty. This analysis and assessment of the data regarding paraquat as a cause of Parkinson's disease is consistent with that of other experts in the field[98] (and "Brief of Amici Curiae J. Timothy Greenamyre, Michael Okun, and Beate Ritz in Support of Petitioners"). Dr. Roger Albin, Director of the University of Michigan Udall Center of Excellence for Parkinson's Disease, and a researcher whose work was foundational to our understanding of the functional anatomy of the brain circuits central to Parkinson's disease states "[t]he case for eliminating or reducing exposure to some industrial toxicants, such as paraquat or halogenated hydrocarbons, is strong. Given the difficulty of studying these

associations, the available data supports causal relationships between these exposures and PD incidence” [99]. Criticism of these conclusions, as exemplified by the manuscript by Weed [54], point to a lack of direct statements in peer reviewed literature stating that paraquat exposure causes Parkinson’s disease. It is important to consider that major journals prohibit the use of causal statements in the absence of a prospective randomized controlled trial in human subjects[100]. Such a trial testing the effects of paraquat exposure on humans would be unethical and dangerous—based on the wealth of information we have about this chemical. With this in mind, multiple researchers in the field of Parkinson’s disease and neuroscience have highlighted the role of paraquat in Parkinson’s disease pathogenesis and the potential benefits of preventing future cases of Parkinson’s disease by eliminating the use of this product [99, 101-103].



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H. References

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