

Paraquat Report October 4, 2024

1. Jason R Richardson MS, PhD DABT Fellow ATS Background and Qualifications

I, Jason R Richardson MS, PhD DABT Fellow ATS, am the Dianne Isakson Distinguished Professor of Physiology and Pharmacology at the University of Georgia College of Veterinary Medicine and the Isakson Center for Neurological Disease Research. I previously served as the Associate Dean for Research, Professor of Environmental Health and Founding Director of the NIH-funded Training in Environmental Neuroscience program at Florida International University (FIU) from 2018-2023. Previous to my appointments at UGA and FIU, I served as the Founding Director for the Center for Neurodegenerative Disease and Aging/Research Focus Area at Northeast Ohio University, while simultaneously serving as Acting Department Chair for Pharmaceutical Sciences, Professor of Pharmaceutical Sciences and Acting Associate Dean for Research (2015-2018). My initial faculty position was at Robert Wood Johnson Medical School (now Rutgers Robert Wood Johnson Medical School) and Resident Member of the Environmental and Occupational Health Sciences Institute (2005-2015). I also served as the Deputy and then Director of the Joint Graduate Program in Toxicology at Rutgers University.

My training includes a Bachelor's degree in General Studies with a major in Toxicology from Northeast Louisiana University (now University of Louisiana at Monroe), a Master's in Veterinary Medical Sciences (toxicology) and a PhD in Environmental Toxicology from Mississippi State University. I further pursued training in Molecular Neuroscience and Neurotoxicology at Emory University where I began my research on environmental factors in Parkinson's disease. I am board certified as a Diplomate of the American Board of Toxicology, where I have been recertified twice, and an elected Fellow of the Academy of Toxicological Sciences.

For more than two decades, I have been the principal investigator or co-investigator on more than a dozen grants focused on Parkinson's disease, pesticides and gene-environment interactions in Parkinson's. I have also received significant funding for my work on gene-environment interactions in Alzheimer's disease and ADHD. As part of these projects, I have led many multi-disciplinary teams to explore the role of environmental exposures in neurological diseases and disorders. These projects include both basic and epidemiological studies.

I have served as a member or Chair for dozens of National Institutes of Health, Department of Defense, Veterans Administration, Michael J Fox Foundation for Parkinson's Disease Research and International grant review panels related to Parkinson's disease, neurotoxicity and pesticides. I Have also served as a member of the NIH Expert Panel Workshop on Toxic Industrial Chemicals, the Michael J Fox Foundation for Parkinson's Disease Research Inflammation Consortium, and the Committee on Emerging Science for Environmental Health Decisions at the United States National Academy of Science (NAS). I am a member for the American Association for the Advancement of Science and the Society of Toxicology (SOT), where I served as Vice President and President of the Neurotoxicology Specialty Section, Vice Chair and Chair of the FDA-SOT Colloquia Committee, and a member of the Committee on Diversity Initiatives. I also served as a member of the Board of Directors for the Academy of Toxicological Sciences. Currently, I Chair the External Advisory Committee for the University of Kentucky Center for Environmental Disease.

I have served as an ad hoc reviewer for over 50 journals, including the Journal of the American Medical Association, Proceedings of the National Academies of Science, Annals of Neurology, Nature Parkinson's Disease and Neurobiology of Disease. I am currently an Associate Editor for several peer-reviewed journals, including Neurotoxicology, Toxicological Sciences, Frontiers in Neurodegeneration, Current Environmental Health Reports and previously, BMC Neurology. I serve on the Editorial Board of several peer-reviewed journals, including, Life Sciences, Toxicology and Applied Toxicology, Neurotoxicology and Teratology and Toxics.

I have published 122 peer-reviewed manuscripts that have been cited over 10,000 times, according to Google Scholar. Two of these manuscripts have been selected as Outstanding Paper of the Year by the National Institute of Environmental Health Sciences. I have also published 16 book chapters and 182 abstracts. I have given over 100 invited presentations nationally and internationally. I have won numerous awards for my research, including the Outstanding New Environmental Scientist Award from the National Institute of Environmental Health Sciences, The Inaugural American Society for Pharmacology and Experimental Therapeutics Toxicology Division Young Investigator Award and the Society of Toxicology Achievement Award.

My curriculum Vitae is Attached.

2. Methodology

Definition of terms:

Weight of Evidence Approach: A weight of evidence approach (WOE) is utilized by the United States Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) for scientific assessments related to associations between chemical exposures and health outcomes (i.e., risk assessment). The World Health Organization also uses this approach. In general, the WOE approach is a process by which all of the relevant evidence for a risk assessment is evaluated and weighted. EFSA has defined this as "a process in which evidence is integrated to determine the relative support for possible answers to a scientific question"¹. The process includes formulating the problem to be addressed, assembly of evidence (i.e., data from multiple recognized sources), weighing the evidence based on quality and consistency, integrating the evidence (i.e., animal and human data), identification of data gaps and areas of uncertainty and ultimately a conclusion based on this process. EPA describes WOE as a collective evaluation of all pertinent information used to adequately consider the full impact of coherence and biological plausibility.

Systematic Review: A systematic review is a synthesis of all relevant and available evidence for review. It requires clearly stated, transparent and reproducible methodology to identify how studies were identified and criteria for inclusion and exclusion of studies from the review.

Meta Analysis: In order to increase statistical power and summarize effects across different studies, a meta-analysis is often performed. A meta-analysis uses the odds ratios or rate ratios along with the confidence intervals from published studies to generate a combined summary estimate of the effect of interest.

Dose-response: A dose-response relationship represents increasing effect on an outcome measure with increasing dose. In mechanistic, typically animal studies, this is observed by treating appropriate numbers of animals with increasing doses of the compound of interest and evaluating the change in a selected outcome measure or measures. For epidemiological studies, a dose-response relationship is typically observed as an increased risk with increased exposure. This exposure can be based on duration of exposure or intensity of exposure.

Unbiased Stereology: Unbiased stereology is considered the gold standard for quantitative histology, whereas qualitative histology has long been the gold standard for routine safety assessment and toxicity studies. It has become increasingly recognized that the human eye is not sensitive enough to detect subtle changes. This is best characterized by a study demonstrating that even trained pathologists could not identify an over 30% reduction in hippocampal neuron number². To overcome this limitation along with several other limitations that introduce bias, more quantitative techniques (i.e., unbiased stereology) are now being employed to increase rigor and accuracy. Stereology does not make assumptions that are inherent in qualitative histology and provides accurate 3-dimensional estimates of volume, number, surface area and length. Because of the greater rigor and accuracy of unbiased stereology, multiple journals, including *Neurobiology of Aging*, have required the use of unbiased stereological techniques for counting of neurons.

Although properly performed unbiased stereology can provide unbiased estimates of several outcome measures, there are issues that relate to precision. In the case of stereology, this precision is related to the variance and reproducibility of the estimate produced. In stereology, this is represented by the coefficient of error (CE) and calculated with the method of Brown and Gunderson³. Typically, the field-standard for CE is less than 10% when properly conducted.

Statistical Significance: In hypothesis-based testing, the initial assumption or null hypothesis is that there is no relationship between the exposure and outcome measure (i.e., paraquat and Parkinson's disease). The p-value does not give the probability that the null hypothesis is true, rather it provides information related to the quality of the evidence in a particular dataset and is highly influenced by sample size, experimental design, analytical procedures and quality of the research. Most often in the literature, a pre-determined p-value, often $p < 0.05$, is considered statistically significant. Although this is the most often used pre-determined value, there can be several others that are more or less stringent. Further, the specific information provided by $p < 0.05$ is related to the random error (i.e., if 100 tests are performed, 5% of the results would be due to random error or chance). Other metrics, including the confidence interval around the mean can often provide additional significant additional information of the relationship between exposure and outcome. For example, the most commonly used estimate is a 95% confidence interval, which indicates that 95% of the time the true result is contained within this interval. It is important to note that statistical significance does not provide biological or clinical significance. For example, a statistically significant effect on a biological protein may not be sufficient to produce a clinical phenotype.

2.1 Literature search

To obtain published studies related to paraquat and Parkinson's disease (PD), I performed literature searches using two well-established search engines, PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) and Google Scholar (<https://scholar.google.com>). These are the two main search engines that I use for the research in my laboratory and those that I train students to use. PubMed is the premier resource for peer-reviewed biomedical research and typically performs exceptionally for papers that clearly identify the topic of the search based on key words. Google Scholar is much broader-based in the search that is performed, often providing ancillary articles. Both search engines were used in order to be as thorough as possible and to identify the most relevant articles. Initially, searches were conducted with the key words of "paraquat" and "Parkinson's disease", which produced 714 studies from 1985-October 2024 in PubMed and Google Scholar produced approximately 18,600 results for the same time frame. To further refine the search to the most relevant questions at hand, results were sorted to focus on animal studies that used unbiased stereological counting of dopamine neurons following paraquat exposure, pharmacokinetic studies of paraquat exposure in animals and on human epidemiological studies and meta-analyses focusing specifically on the relationship between paraquat and Parkinson's disease.

2.2. Peer-reviewed literature

Peer-review can be defined as a process in which manuscripts submitted for publication are reviewed by multiple referees outside of the institution or place of work of the authors. Most often, the reviewers are experts in the field of study related to the manuscript. Reviewers are responsible for evaluating the manuscript for originality, significance, rigor and whether or not the manuscript is appropriate for the particular journal to which it was submitted. The overall goal of peer review is to assess the quality of the data presented in the manuscript, identify flaws and areas of improvement and to ensure only the most rigorous and meritorious manuscripts are published. In addition to evaluating the data reported in the manuscript, the reviewers are also tasked with assessing methodology and the conclusions of the manuscript. Ultimately, reviewers are asked to provide their recommendation on whether the paper should be accepted in its current form, which is exceedingly rare, whether the manuscript requires minor or major revisions, or should be rejected. The Associate Editor and/or Editor of the Journal ultimately has the final say, but typically follow the recommendation of the reviewers.

Peer-review is considered the gold standard of science, both in manuscripts and competitive grant applications and has been practiced since the 1970s. The vast majority of reviewers are anonymous in the majority of scientific journals, while the authors are typically identified. I have personally reviewed hundreds of manuscripts for over 50 journals in the areas of toxicology, neuroscience, neurology, pharmacology, epidemiology and biochemistry. I have also handled, which includes assigning reviewers and making final decisions on acceptance, revision or rejection, of hundreds of manuscripts for multiple journals that I have served as an Associate Editor.

2.3 Overall Methodology: In preparing this report and forming my opinion on the causal relationship between paraquat and Parkinson's disease using a WOE approach. There are several different approaches to WOE analysis^{1,4}. Typically, a formal full WOE approach includes

multiple contributors of varying backgrounds. However, I have used the general framework of WOE for my evaluation of animal and epidemiological studies regarding paraquat and its potential relationship to Parkinson's disease. Based on the types of WOE assessments illustrated by Linkov and co-workers⁴, I have used the "Best Professional Judgement", which involves qualitative integration of multiple lines of evidence. I have relied almost exclusively on peer-reviewed literature. I performed my own review of the data reported in the literature and some reports or data provided by Counsel that are Syngenta documents. Specific to this report, the lines of evidence considered are animal studies involving paraquat's effects on dopamine neuron loss in the substantia nigra, as evidence through unbiased stereological counting, pharmacokinetic studies and human epidemiological studies, including systematic reviews and meta-analyses, that specifically explore the relationship between paraquat exposure and risk of Parkinson's disease.

3. Literature Review

Below, I will provide a brief introduction to Parkinson's disease etiology and pathogenesis. I will then summarize my findings on animal studies related to paraquat pharmacokinetics, the relationship between paraquat exposure and dopamine neuron loss followed by epidemiological studies focusing on the relationship between paraquat exposure and Parkinson's, with particular emphasis on recently published meta-analyses.

Parkinson's Disease (PD) is the second most common neurodegenerative disease, behind only Alzheimer's disease, and is estimated to affect over 10 million people worldwide, including over 1 million people in the United States. Age is the largest risk factor for developing PD, with 1% of the US population over the age of 65 developing PD. The prevalence of PD is inevitably going to rise dramatically over the next 20 years with the number of Americans over 65 rapidly increasing⁵. The major clinical presentations of PD include motor dysfunction primarily presenting as bradykinesia, muscular rigidity, a resting tremor, and postural instability. Although many neurotransmitter systems are now known to be disrupted in PD, these cardinal signs are primarily the result of selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) which send dopamine to the striatum.

Although genetic factors are thought to be significant contributors to the etiology of PD, studies to date demonstrate that less than 15% of PD cases are the result of single gene mutations⁶. The role of the environment in the pathogenesis of PD was supported by twin studies showing that the concordance of PD was virtually identical in monozygotic and dizygotic twins^{7, 8}, and the discovery of MPTP, which is a byproduct of opioid drug manufacturing and caused an acute and permanent parkinsonian syndrome⁹. MPTP easily crosses the blood-brain barrier and is converted to the active neurotoxin MPP⁺, and MPP⁺ results in selective loss of dopamine neurons and striatal dopamine, similar to that observed in PD, by selectively inhibiting mitochondrial complex I of the electron transport chain of dopaminergic neurons¹⁰. Although evidence clearly supports the involvement of the environment, no specific environmental toxin or toxicant has been unequivocally linked to the development of PD. Multiple epidemiological studies support the potential role of environmental factors in the etiology of sporadic PD^{11, 12}, including extensive evidence that individuals exposed to pesticides have increased risk for PD¹³⁻²⁴.

Epidemiological studies have linked exposure to one particular pesticide, the herbicide paraquat (PQ), to an increased risk of PD^{23, 25-27}. Recent meta-analyses have confirmed these individual reports and demonstrate an estimated increased risk of approximately 1.25-1.75^{24, 28, 29}. This is particularly important, as PQ usage has increased by over 3X in the past 15 years in the United States and 2017 data from the US Geological Survey report that approximately 17 million lbs of PQ were applied for agricultural use

(https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2017&map=PARAQUAT&hilo=H). Further, numerous publications demonstrate that injection of rodents with PQ results in hallmarks of PD, including increased α -synuclein levels, oxidative damage, neuroinflammation and loss of a subset of dopamine neurons³⁰⁻³⁵. Therefore, the weight of the evidence clearly demonstrates that PQ injection results in death of dopamine neurons in the substantia nigra, along with other aspects associated with Parkinson's disease, and these findings support epidemiological data linking PQ exposure to PD. Below, I will discuss specific evidence related to the ability of paraquat to enter and persist in the brain, animal studies demonstrating effects of paraquat on dopamine neuron number and epidemiological studies related to paraquat exposure and Parkinson's disease.

3.1 Animal pharmacokinetic studies demonstrating that paraquat enters the brain

Based on the charged nature of the paraquat molecule, it was long thought in the scientific community that paraquat was unlikely to be able to cross the blood-brain barrier and enter the brain³⁶. However, there was early evidence in high exposure/suicide cases that paraquat could be found in the brains of humans.⁸⁵ Subsequent animal work demonstrated that paraquat likely enters the brain through the neutral amino acid transporter^{34, 37, 38} (and possible through the organic cation transporter 3).

As early as 1976, Syngenta published data demonstrating that brain and lung slices accumulated radiolabeled paraquat and that rats dosed with radiolabeled paraquat demonstrated measurable brain levels of paraquat, although not to the extent of other tissues³⁹. Subsequent Syngenta studies that used radiolabeled paraquat and exposed brain slices from the brain to film argued that there is limited entry to the brain^{40, 41}. Unfortunately, autoradiography using carbon-14 labeled compounds is a relatively insensitive technique compared to more modern analytical chemistry techniques. As analytical techniques became more sensitive, the use of liquid-chromatography coupled to mass spectrometry allowed for detection of paraquat in animal plasma and brains⁴²⁻⁴⁴. Data from the Breckenridge study⁴⁴ (Syngenta) demonstrated that peak plasma concentrations were reached rapidly and were reduced to background levels within hours. However, the studies by Prasad et al. and the Breckenridge study generated similar estimates of the half-life of paraquat in the brain of 22.5-28 days⁴²⁻⁴⁴ and reported that paraquat was linearly accumulated in the brain following multiple doses. This finding of long-term presence and accumulation of paraquat raises concerns related to the neurotoxicity of paraquat discussed below. A Syngenta study from Minnema et al.⁴⁵ administered paraquat in the diet of mice for 13 weeks. This study also found cumulative increases in brain levels of paraquat and a protracted residence of paraquat in the brain.

The studies discussed in the prior paragraph were all based on intraperitoneal, intravenous or oral exposure. Dermal and inhalation exposure are also relevant exposure paradigms for people exposed to paraquat. There are no published pharmacokinetic data in the literature, but an Exponent report

to the Department for Environment and Rural Affairs that was mainly focused on the combination of paraquat and maneb did report pharmacokinetic data in adult mice for technical paraquat and a commercial Gramoxone (paraquat) formulation for intraperitoneal and oral, dermal and intranasal administration, respectively.⁸⁶ The report was inconsistent in 2 places, with one note saying only technical paraquat was used for intraperitoneal studies and another note saying that Gramoxone was used. All formulations were radiolabeled for detection. In Figure 1, curves of blood and brain levels of paraquat after a 10 mg/kg oral exposure are provided and match well with the persistence of paraquat in the brain observed in the published studies above, although only 3 mice were used. The dermal data (40 mg/kg paraquat/Gramoxone) were not considered as relevant because rodent skin is a poor surrogate for human skin and the to replicate field conditions, a surfactant was not included. The surfactant issue is extremely important, as data have demonstrated that surfactant levels can increase paraquat penetrance and increase internal levels from a variety of routes of administration, including studies from Syngenta. Further, the authors noted that the dermal data showed the most variability and not described well by the pharmacokinetic model. What is likely the most important and relevant finding of the study is that intranasal exposure resulted in orders or magnitude higher brain levels than the oral or intraperitoneal route.

Another study by Anderson et al.⁴⁶ used whole-body inhalation exposure of aerosolized paraquat in mice to determine brain levels of paraquat. Although this was not designed as a pharmacokinetic study and there was non-linearity between timepoints, the data demonstrate that there is protracted accumulation and persistence of paraquat in the brain over days and weeks. It further showed accumulation of paraquat was highest in the olfactory bulb, but reached other areas in the brain, including the midbrain that contains dopamine neurons. Rojo et al.⁴⁷ inoculated paraquat into the nasal cavity at a single dose of 20 mg/kg and measured paraquat levels. Unfortunately, the measurement was only taken at one timepoint and that was 10 minutes after inoculation, which is a significant weakness of the study. Not surprisingly, the authors only detected paraquat in the olfactory bulb and not in the striatum or midbrain. Collectively, these two studies demonstrate that paraquat can reach the brain through inhalation or intranasal distillation.

Rodents have a much higher metabolic rate and more rapid physiological processes than primates, which is associated with more rapid excretion of drugs and chemicals⁴⁸. This was confirmed by a Syngenta pharmacokinetic study that demonstrated that the half-life of paraquat in the plasma of non-human primates was 143 hours⁴⁹ compared to the rapid return to baseline levels within 4-5 hours in the mouse⁴⁴. With regard to the brain, one study used carbon-11 labeled paraquat in rhesus macaques and positron emission tomography to explore the brain uptake of paraquat following intravenous administration in anesthetized macaques⁵⁰. This study reported little uptake of paraquat with the exception of the pineal gland and lateral ventricles. Unfortunately, the use of ketamine to anesthetize the macaques is a significant confounder, as ketamine is known to inhibit the neutral amino acid transporter and is a substrate of multiple organic cation transporter transporters, including OCT2, which can transport paraquat in kidney cells and is present in the brain and OCT3 that has been linked to transporting paraquat into the brain^{34, 37, 38}, thus competing for uptake and likely reducing the levels of paraquat in the brain. A subsequent Syngenta study unfortunately used the entire carcass rather than specific organs and tissues to measure radioactive paraquat levels⁴⁹. Thus, the amount of paraquat in the brain could not be determined. I have also been provided data generated by Syngenta using LC/MS to measure brain levels of paraquat in

squirrel monkeys treated subcutaneously with paraquat by Dr. Di Monte.⁸⁷ These data clearly show measurable brain levels of paraquat. Further, the brain levels did not change from 1 week to 8 weeks post-exposure, suggesting that the half-life of paraquat in the brain of the primate is orders of magnitude longer than rodents. It should be noted that no half-life could be calculated from these data, as there were no decreases in brain paraquat levels at the last time point, 8 weeks post exposure. Therefore, it is not clear how long paraquat ultimately resides in the non-human primate brain, but it is clearly longer than 8 weeks.

Collectively, the pharmacokinetic data clearly demonstrate that paraquat can enter the brain and has a prolonged residence time compared to the plasma via various routes of paraquat administration. Further, primates appear to retain brain levels of paraquat far longer than rodents. Additionally, Finally, in multiple animal species, the accumulation of paraquat in the brain is linear. These findings have significant implications for neurotoxicity, as toxicity is the accumulation of damage over short or long period of time and is a function of exposure, which is the result of dose and time⁵¹.

3.2 Animal studies reporting paraquat effects on dopamine neuron number using unbiased stereological methods.

In my review of studies involving paraquat administration and dopamine neuron loss, I restricted my analysis to those studies that performed experiments in adult animals (almost exclusively mice in the literature), studies that involved paraquat administered alone and not in combination with other chemicals or agents, and studies that used unbiased stereological counting to quantify dopamine neuron loss.

The initial report of the potential effects of paraquat on dopamine neurons in the substantia nigra was published by Brooks et al.³⁰ This paper showed an apparent decrease in dopamine neurons, but used a retrograde tracing technique and counts were not performed using unbiased stereology, reducing confidence in the findings. The first manuscript reporting the effects of paraquat on dopamine neurons using unbiased stereology was published in 2002 from Dr. Dino Di Monte's laboratory³². In this manuscript, male C57 BL/6 mice of 6 weeks, 8 weeks, 6 months and 18 months of age were administered 3 intraperitoneal injections of saline or paraquat once a week for 3 consecutive weeks and sacrificed 7 days after the last treatment. Unbiased stereology was used to count dopamine (i.e., tyrosine hydroxylase positive (TH+) neurons in the substantia nigra) and total neurons, as assessed by nissl staining. The manuscript reported a dose-response relationship where doses of 1, 5 and 10 mg/kg paraquat resulted in 10, 18 and 28% loss of TH+ neurons. This loss was increased to 33% in the 18-month-old mice, with no differences observed between 6 week or 6-month-old mice. Importantly, the loss of TH+ neurons was accompanied by a similar decrease in nissl+ (total neurons), indicating that there was a true loss of neurons and not simply loss of TH expression. Using a similar paradigm, further studies by this group replicated this initial finding repeatedly^{38, 52-55}.

Subsequently, additional studies from the Andersen laboratory that dosed mice with 7 mg/kg paraquat every other day for 10 total doses by intraperitoneal injection observed a 28% loss of TH+ neurons in the substantia nigra^{56, 57} and an additional study where mice were administered paraquat (10 mg/kg via intraperitoneal injections) twice per week for 3 weeks⁵⁸. Several other groups have replicated these findings using similar dosing paradigms^{34, 59-62}. Thus, there is significant and

consistent evidence in the literature that injection of paraquat to mice results in loss of dopamine neurons in the substantia nigra.

Conversely, there are 3 papers in the literature using unbiased stereological methods that report no significant effect on dopamine neuron numbers. The first was a Syngenta-sponsored study published by Breckenridge and co-workers in 2013⁴⁴. This manuscript used several different intraperitoneal injection paradigms to reproduce the various paradigms used in the studies cited in the previous paragraph. They also included a MPTP group as a positive control (4 doses of 10 mg/kg every 2 hours). The manuscript reported that administration of paraquat at 3 doses of 10 mg/kg, 1 dose of 15 mg/kg and 2 doses of 15 mg/kg paraquat did not result in statistically significant decreases of TH+ neurons. Administration of 3 doses of 15 mg/kg did result in statistically significant decrease in TH+ neurons and administration of 3 doses of 10 mg/kg of paraquat supplied by Sigma-Aldrich resulted in a loss of TH+ neurons that did not quite reach statistical significance ($p=0.068$). In none of the studies were cresyl violet stained neurons shown to be significantly decreased, suggesting no neuronal loss. There are, unfortunately, many significant problems with this study that raise concern to the validity of the findings. The first is that the MPTP positive control did not result in loss of total neurons and did not produce significant TH+ cell loss in all studies (Study 4, significant vs Study 5, significant with fluorescent counting, but not chromogenic staining). Further, it appears that the MPTP treatment in the one study that did show a statistically significant result resulted in a loss of around 30% TH+ neurons, whereas literature studies by others show that this treatment paradigm results in approximately 50% loss of TH+ neurons and total neurons (nissl+)⁶³. The second is the previously discussed internal inconsistency was not replicated (i.e., Study 4 vs Study 5). As noted by the authors and in another manuscript that included some of the same authors⁶⁴, the coefficient of error was much larger in this study (all over 0.12), demonstrating imprecise measurement in this study. Indeed, the authors acknowledge that "it is possible that the stereological methods used in our studies were inadequate to detect a PQ-induced reduction in the number of dopaminergic neurons reported by others". Based on the high coefficient of error, of which the field standard is less than 0.1, I agree with the authors. Furthermore, previously unpublished and unreported studies by Syngenta conducted by Marks after she trained in stereology with the Di Monte group was able to replicate the effects of paraquat in reducing dopamine neurons.⁸⁸ It is not clear why this was not mentioned in this manuscript.

The next two studies were also supported by Syngenta. The first was published in 2014 by Minnema et al⁴⁵. This was a 13-week study in which animals were fed chow containing 10 or 50 parts per million of paraquat, resulting in dose levels of 2.4 and 14.1 mg/kg body weight/day for males and 1.7 and 3.7 and 21.5 mg/kg body weight/day for female mice. As with the Breckenridge study (2013), MPTP was used as a positive control and also observed a much lower decrease in dopamine neurons (~10%) following MPTP administration in male mice and a non-significant decrease of 5% in female mice. This is in fact much lower than that what appears to be reported in the Breckenridge paper for the same doses of MPTP. Also similar to the Breckenridge paper, no significant effect of paraquat was observed. Based on the brain levels in the study being very similar to that produced by the 3x15 mg/kg dosing of paraquat described in the Breckenridge paper that also showed that this dose produced dopamine neuron loss, there is decreased confidence in this study. Although the coefficient of errors are not reported in this manuscript, it is likely they are similarly high as the Breckenridge paper, as it appears the stereology was performed by the same contractor.

The final paper is another Syngenta study in collaboration with Smeyne, who was at that time an investigator at St. Jude Children's Research Hospital⁶⁴. MPTP was again used as a positive control, but with a higher dosing schedule of 4x20 mg/kg MPTP. This is a dose known in the literature for producing ~70% loss of dopamine neurons. Paraquat was administered once a week for 3 weeks at 20 mg/kg or twice per week at 10 mg/kg. All treatments were given via intraperitoneal injection. In Figure 1 of the manuscript, MPTP appears to only decrease TH+ neurons by 40%, much lower than expected from the literature. Further, the Figure is labeled as 4x16 mg/kg MPTP. So, it is not clear what dose of MPTP was actually used for these studies. For the paraquat studies, no significant loss of dopamine neurons was observed. This is very surprising because a previous study by Smeyne's group using the same dosing regimen of 10 mg/kg paraquat twice a week for 3 weeks observed a significant 48% loss of TH+ neurons⁶². Inspection of the "systematic review" in this paper revealed that of the almost 50 studies considered, only 3 studies did not show dopamine neuron loss when considering similar dosing paradigms and studies that performed unbiased stereological counting of neurons. These were studies sponsored by Syngenta^{44, 45, 64, 65}. Further, previously, undisclosed studies by Marks at Syngenta demonstrated dopamine neuron loss similar to the published literature following training in Di Monte's laboratory in unbiased stereological counting (XM7258, XM7371, XM7480, SYNG-PQ-00116782 SYNG-PQ-13083935SYNG-PQT-ATR-00963849 SYNG-PQ-01977520).⁸⁸ This was not mentioned in any of the 3 Syngenta papers.

There are many fewer studies in the literature for rats that used unbiased stereology. Ossowska et al. reported that weekly intraperitoneal administration of 10 mg/kg paraquat resulted in a significant loss of 37% of TH+ neurons after 24 weeks⁶⁶. A 17% loss of TH+ neurons was observed following 4 weeks of treatment and 28.5% loss after 8 weeks of treatment, but these barely missed statistical significance ($p=0.1$ and $p=0.06$, respectively). In another study by the same group, daily subcutaneous injections of paraquat (10 mg/mg) once a day for 5 days and sacrificed 3 days after the last injection. This treatment paradigm resulted in a 22% loss of TH+ neurons. A study by Cristovao et al. employed subcutaneously implanted mini-pumps to deliver 2.5 mg/kg/day of paraquat for 4 weeks and sacrificed 4 weeks later. A 41% loss of TH+ neurons were observed in this model⁶⁷. Because of the use of the mini-pump to deliver sustained doses of paraquat rather than the intermittent exposures by injection, I gave this study less weight. Anselmi et al. administered paraquat (1 mg/kg) in combination with lectin for 7 days and observed significant loss of TH+ neurons.⁶⁸ The authors state in the publication that the paraquat alone group did not show a significant decline in TH+ neurons, but the data were not shown in the manuscript and the number of control animals was exceedingly low (3 control animals and 5 paraquat animals). Because of these two issues, I gave less weight to this study.

There have been two studies involving intranasal administration of paraquat^{47, 69}. Unfortunately, neither of these studies used unbiased stereology for counting dopamine neurons and were not considered further.

Overall, the weight of the evidence in the animal studies demonstrates that there is a clear and consistent reduction of dopamine neurons in mice and rats following administration of paraquat.

3.3 Epidemiological Studies

Early epidemiological studies on environmental factors and risk of Parkinson's disease pointed to rural living, consumption of well water and farming as significant risk factors. Because of this, occupational and environmental studies started to explore the relationship between pesticide exposure and Parkinson's⁷⁰. The first peer-reviewed study that specifically evaluated the relationship between paraquat exposure and Parkinson's was a case-control study in Taiwan by Liou et al.²⁵ This study utilized movement disorder specialists to diagnose Parkinson's and had good exposure metrics. It reported that the use of paraquat, particularly increasing lifetime use, was associated with significantly increased risk of Parkinson's disease (Odds Ratio (OR) = 3.2, 95% CI = 2.41-4.31 for reported use of paraquat and OR = 6.41, 95% CI = 2.74-15.00 for those occupationally using paraquat for more than 20 years).

Tanner et al. conducted a nested case-control study within the National Institutes of Health supported Agricultural Health Study, which is the largest cohort study of pesticide use in the United States²³. Licensed pesticide applicators, primarily farmers, and their spouses (84,739) were recruited and enrolled in North Carolina and Iowa from 1993-1997. The Tanner study enrolled 115 Parkinson's patients and 383 controls and reported a significant association between paraquat exposure and Parkinson's disease (OR = 2.5, 95% CI = 1.4-4.7). This paper was weighed heavily because of the use of movement disorder specialists to diagnose Parkinson's (consensus review by 2 movement disorder specialists), reducing the likelihood of misdiagnosis and misclassification. There was also significant granularity in the exposure data and the conclusions were confirmed by multiple sensitivity analyses performed by an external group (Exponent) that was contracted by Syngenta.⁸⁹ Another case-control study in a subset of patients from the Tanner study²³ with 87 Parkinson's subjects and 343 matched controls found that Parkinson's risk was higher from paraquat in those with a genetic deletion of glutathione transferase T1 (GSST1), which is important for protecting against oxidative stress. In men with the full deletion of GSST1, there was an OR of 11.1 (95% CI = 3.0-44.6)⁷¹. The Liou and Tanner studies collective provide strong support for the association between occupational exposures to paraquat and risk of Parkinson's disease.

Several, case-control studies by Ritz as part of the Pesticide, Environment and Genes Studies (PEG) also demonstrate significant strengths including the use of the California pesticide use reporting records in the place of self-reporting and the use of movement disorder specialists to confirm diagnosis. Exposures are based on a validated geographic information system model⁷². In the first paper from this study⁷³, ever/never use of paraquat near a residence was not associated with an increased risk of PD (OR = 1.01, 95% CI = 0.71-1.43). In contrast, a follow up manuscript⁷⁴ found that paraquat exposure at the residence and workplace did demonstrate a significantly increased risk for Parkinson's (OR = 1.5, 95% CI = 1.03-2.18), suggesting workplace/occupational exposures likely drive the increased risk of Parkinson's from paraquat exposure. In a larger follow up (Lee et al., 2012), this effect was confirmed (OR = 1.36, 95% CI = 1.02-1.81) and demonstrated an even further enhancement of risk if the exposure was coupled with another Parkinson's disease risk factor, head injury (OR = 3.01, 95% CI = 1.51-6.01). Additional studies by this group reported enhancement of paraquat exposure with Parkinson's based on several genetic susceptibility variants^{75, 76}. Most recently, Ritz's group reported the largest case-control study to date on the relationship between paraquat and Parkinson's disease⁷⁷. A total of 829 Parkinson's patients and 824 community matched controls in the farming region of Central California and had an average exposure timeframe of ~31 years, which allowed for determination of both duration and intensity of exposures. Estimating

workplace proximity to commercial application of paraquat since 1974, the group found increased odds of PD using ever/never use of PQ, along with duration and intensity of exposure. For the intensity of exposure, there was an estimated OR of 2.08 (95% CI = 1.31-3.38) per 10 lbs paraquat active ingredient applied per acre in a designated buffer area from 1974 when using the work address. Similar to previous findings from this group, intensity of paraquat exposure related to risk of Parkinson's at the residential address was lower than the workplace, but still elevated (OR = 1.72, 95% CI = 0.99-3.04). Looking at duration of exposure, the group found similar results. Collectively, the Ritz studies clearly demonstrate that intensity and duration of paraquat exposure significantly increase risk of Parkinson's.

There are also several case-control studies that do not find an association between paraquat and Parkinson's disease^{26, 78-81}. Unfortunately, these studies do not have as rigorous and granular exposure metrics and most suffer from having few participants with paraquat exposure. Therefore, these studies were not weighed as heavily. However, these are included in the meta-analyses that are discussed in the next paragraph.

As mentioned in the definitions above, in order to increase statistical power and summarize effects across different studies, a meta-analysis is often performed. These are particularly important for studies with paraquat and Parkinson's disease, as many of the studies have smaller numbers of people exposed to paraquat. To date, there have been 4 published meta-analyses that explored the relationship between paraquat and Parkinson's disease. Pezzoli and Cereda⁸² found an overall significantly increased risk of paraquat and PD of 2.19 (95% CI = 1.48-3.26) based on 7 studies and 1.72 (95% CI = 1.28-1.32) when restricting to the 5 studied identified as the highest quality. Tangamornsukan et al.²⁸ selected 13 case-control and 1 cohort study and used all of the case-control studies for the final meta-analysis. These studies were comprehensive and included studies from 7 different countries around the world. The meta-analysis found an overall OR of 1.64 (95% CI = 1.27-2.13) for the association between paraquat exposure and Parkinson's disease. Several sensitivity analyses were also performed and confirmed the initial results. In 2019, Vaccari et al.²⁹ identified 11 studies and reported an OR of 1.43 (95% CI = 1.06-1.91) for ever/never use of paraquat and Parkinson's disease. Finally, a Syngenta meta-analysis²⁴ reported similar results as the other 3 studies. They reported a random-effects OR of 1.47 (95% CI = 1.01-2.13) for what the authors considered their top 2 tiers of studies. Additional analysis with a fixed-effects model that included all studies reported a risk ratio of 1.69 (95% CI = 1.44-1.98). The consistency of these 4 studies is weighed highly, as each set out a slightly different set of conditions for the meta-analysis and gave different weights to various studies included in all of the papers.

It should be noted that additional relevant studies have been published since the last meta-analysis in 2019. The latest study from the PEG cohort⁷⁷ was discussed above and provided strong support for the association of paraquat with Parkinson's disease. The second relevant study was a more recent follow up case-control study out of the Agricultural Health study⁸³ involving 373 farmers and 118 spouses that self-reported that a doctor had diagnosed them with Parkinson's disease. This study did not find a statistically significant increased risk of Parkinson's with paraquat exposure (OR = 1.09, 95% CI = 0.84-1.41) for ever paraquat use. Unfortunately, there are several issues with this paper that resulting in reduced weight being given. First, there is an enormous amount of loss to follow up, with 36% of male farmers lost at the first follow up and 54% lost at the second follow up. This loss has multiple profound effects. First, with a disease like Parkinson's that is fairly rare, and

this loss precludes determination of Parkinson's due to the lack of Parkinson's disease registries in Iowa and North Carolina and that Parkinson's is often not noted on death certificates. The second is there is a loss of updated exposure data. This is particularly important, as it is cited above that the use of paraquat has exponentially increased since 2004 and this study primarily relied on data collected at enrollment or a second survey that occurred from 1999-2004. Further, there was reliance on a questionnaire that was only returned by 44% of the farmers. Indeed, the authors noted that "We cannot rule out the possibility that limited evidence of independent associations between Parkinson's disease and ever-use of some pesticides (including paraquat) in the study resulted from non-differential bias attenuating HR estimates". Although the authors conducted a sensitivity analysis using weighted Cox models, the denominator of the weights included the probability of overall participation in Phase 4 based on exposure, year and baseline covariates, with the numerator being the probability of overall participation in Phase 4 depending on year. The assumption underlying this approach assumes that the outcome is not influenced by exposure or other variables and that the earlier exposure measures are adequate indicators of the continued exposure. This is problematic because paraquat use started drastically increasing in the United States in 2004. Others have recently commented on this paper as well⁸⁴ (Darweesh et al., 2024) and raised concerns that paraquat-exposed subjects may have dropped out of the study due to adverse health effects. Thus, the sensitivity analysis likely did not address these issues and the overall result may be biased.

Overall, the weight of the evidence from the strong case-control studies and the highly consistent meta-analyses provide clear and strong support for increased risk of Parkinson's disease following paraquat exposure. It further appears that longer duration.

4. Summary and Conclusion

The weight of the evidence from combination of pharmacokinetic, animal studies exploring the key pathology of Parkinson's disease (i.e., dopamine neuron loss) and the epidemiological studies as a whole strongly support paraquat as causative factor for Parkinson's disease in exposed individuals, particularly those in occupational settings where there is higher intensity and longer duration exposure to paraquat. I hold all of the opinions expressed in this report to a reasonable degree of medical and scientific certainty.



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10/04/2024

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