EXAMINING ASSOCIATIONS BETWEEN GLYPHOSATE EXPOSURE AND DNA ADDUCTS IN OCCUPATIONALLY-EXPOSED ORCHARD WORKERS

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ABSTRACT

EXAMINING ASSOCIATIONS BETWEEN GLYPHOSATE EXPOSURE AND DNA ADDUCTS IN OCCUPATIONALLY-EXPOSED ORCHARD WORKERS

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Glyphosate is a widely used herbicide. In 2015, The World Health Organization's International Agency for Research on Cancer (IARC) changed the category of glyphosate's carcinogenic potential from 'possible carcinogen' to 'probable carcinogen'. Occupationally exposed workers in the agricultural industry may be at a higher risk of developing cancer due to glyphosate exposure, particularly if workers do not adhere closely to personal protection guidelines. The IARC based the glyphosate reclassification largely on experimental animal models, but additional human studies are needed to determine association and potential causation between glyphosate exposure and increased cancer risk. The challenges posed by human studies include the long follow-up and rarity of cancer in prospective studies and the difficulty of getting accurate exposure measures in retrospective case-control studies. The following literature review and R03 establishes rationale for the need for this research will examine the association between glyphosate exposure in orchard herbicide sprayers and DNA adducts post-exposure, a proxy for future cancer risk. The goal of this small-scale study is to test logistics and generate data that would inform methods for a larger study of glyphosate exposure and DNA adducts among orchard workers.

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Introduction

Glyphosate, also known by the trade name Roundup, is a broad-spectrum herbicide widely used in the United States. Glyphosate kills plants and bacteria by inhibiting the bacterial and plant enzyme enolpyruvylshikimate-phosphate synthase (EPSPS). The company Monsanto, the maker of Roundup, has developed a way to introduce a bacterial gene for a glyphosate-resistant EPSPS into plants, so that glyphosate can be used for weed control on otherwise glyphosate-susceptible crops [1].

As of 2014, glyphosate accounted for 90% of agricultural herbicide use. Glyphosate was introduced in 1974 and was not widely used until 1996 when genetically engineered soybeans, corn, and cotton were developed to resist glyphosate. Glyphosate use increased from 12.5 million pounds in 1995 to 250 million pounds in 2014, a 20-fold increase [2].

Glyphosate is in a class of chemical substances known as organophosphates. Exposure to organophosphates has been associated with disruption of acetylcholinesterase (AChE) in the central and peripheral nervous system. AChE inhibitors can be divided into two groups: temporary (therapeutic uses in pharmacology) and irreversible (such as from organophosphorus compound exposure) [3]. Oxidative stress from AChE reduction may lead to genotoxicity from both acute and chronic exposure. Oxidative stress is an imbalance between reactive oxygen species, also known as free radicals, and the antioxidant defense system. Organophosphate induced free radicals attack lipids, proteins, and DNA; within DNA the free radicals cause single and double strand breaks [4].

Exposure to glyphosate through agricultural occupation routes may include faulty spraying equipment, accidental spills, ignoring personal protection guidelines, and windy

conditions while spraying [5]. Personal protection guidelines for Roundup PRO and Roundup PROMAX include long pants and shirts, closed-toe shoes, gloves and eye protection but do not require or recommend breathing protection [6]. The Environmental Protection Agency Worker Protection Standard does require the use of respirators, but owners of agricultural establishments and immediate family members are exempt from complying with the standard [7].

In August 2016, the International Agency for Research of Cancer (IARC), under the World Health Organization (WHO), published an updated monograph on glyphosate. This included rationale for upgrading their classification of glyphosate from possible carcinogen to probable carcinogen [8]. There are five categories of carcinogenic levels in IARC monographs, identified as follows [9]:

Group 1	*Carcinogenic to humans*
Group 2A	*Probably carcinogenic to humans*
Group 2B	*Possibly carcinogenic to humans*
Group 3	*Not classifiable as to its carcinogenicity to humans*
Group 4	*Probably not carcinogenic to humans*

The updated glyphosate monograph examined cohort, case-control, and experimental in vivo animal and in vitro (human cells) glyphosate exposure studies. Exposure in cohort and case-control studies was defined by ever personally mixing or applying products containing glyphosate and was quantified by cumulative lifetime days of use. Glyphosate was not associated with melanoma, multiple myeloma, Non-Hodgkin Lymphoma, leukemia, and cancers of the oral cavity, colon, rectum, pancreas, kidney, bladder, or prostate, when analyzed

collectively, but was significantly associated with some of these cancers analyzed individually [10].

Epidemiological and Experimental Studies Assessing Glyphosate as a Carcinogen

Glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), induced genotoxicity in studies of human cells (in vitro and in vivo) and laboratory animal studies that included glyphosate exposure routes through ingestion or injection [10]. Epidemiological studies observed of community-based exposure found positive associations between aerial spraying with glyphosate and DNA damage two weeks to two months after spraying [10]. Six case-control or cohort studies assessed risk of various cancers in relation to glyphosate exposure (Table 1). L. Fritshi *et al* used self-report, which is a study weakness, to determine level of exposure and found the highest odds ratio (OR=3.09) for Non-Hodgkin Lymphoma (NHL) [11]. De Roos *et al* used the Agricultural Health Study, a prospective cohort with over 50,000 participants, and found no association between glyphosate exposure and NHL. However, even this large cohort may not be large enough to study NHL due to it being a rare disease with a U.S. incidence of 3/100,000 [12]. The second De Roos *et al* study pooled data from multiple case-control studies, making it hard to know the impact of recall bias and timing of exposure and disease onset. Furthermore, if a study subject had a missing value for any one of the 47 pesticides evaluated, that person was excluded from analyses, resulting in analyses on a limited subset (about 75%) of the pooled study population. This may have created some selection bias and decreased study statistical power [13]. Hardell *et al* relied on self-reports of exposure and did not adjust for potentially important covariates of lifestyle and health history

[14]. Chang and Delzell's *et al's* systematic review and meta-analysis, funded by Monsanto, reported a slight positive association between glyphosate exposure and NHL, though much of the discussion was dedicated to the individual studies' flaws [15]. Chang and Delzell's *et al* systematic review and meta-analysis shows consistency, since the combined studies used different methods but a slightly positive association was found. Sorahan's *et al* study, which focused on multiple myeloma, had a relatively small number of cases [16].

Table 1: Cohort and case control studies with occupation or glyphosate as exposure and various cancers as outcome

Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[11] L. Fritshi *et al* 2005	Occupational Exposure to Pesticides and Risk of Non Hodgkin Lymphoma	Case-control	Occupation	Non Hodgkin Lymphoma	Cumulative Time and Amount of pesticide exposure, age, sex, region of residence	Exposure to a substantial amount of any pesticide including glyphosate produced a 3x increase of NHL risk. OR 3.09, CI 1.42, 6.70.	Participants were from New South Wales, Australia. Cases and Controls were matched 1:1.
Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[12] De Roos *et al* 2005	Cancer Incidence among Glyphosate-Exposed Pesticide Applicators in the Agricultural Health Study	prospective cohort	Pesticides	Non Hodgkin Lymphoma	level of glyphosate exposure, age, education, smoking status, alcohol consumption, family history of cancer in 1st generation family members, exposure to other pesticides	No significance found in NHL associated with glyphosate exposure among participants of all exposure levels	
Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[13] De Roos *et al* 2003	Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's Lymphoma among men	Pooled analysis of 3 case control studies	47 various pesticides	Non Hodgkin Lymphoma	race, age, pesticide class, structure, known toxicity, occupation	Glyphosate was associated with a slight increase risk of NHL: OR 1.3	

Table 1 (cont'):							
Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[14] Hardell *et al,* 2001	Exposure to pesticides as a risk factor for NHL and HCL: Pooled Analysis of 2 Swedish case-control studies	case-control	various pesticides	Non Hodgkin lymphoma and Hairy cell leukemia	time from last exposure to diagnosis	Glyphosate univariate OR: 3.04 CI 1.08, 8 52 / Multivariate OR: 1.85 CI 0.55-6.20	Study didn't seem to adjust for usual covariates
Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[15] Chang and Delzell 2013	Systematic review and meta-analysis of glyphosate exposure and risk of lymphohematopoietic cancers	systematic review and meta analysis	glyphosate	Non Hodgkin Lymphoma	vital statistics, smoking, family cancer history, non-job related exposure, state of residence, education, gender, history of infectious disease, exposure to hair dyes, SES, exposure to animals, alcohol	Combined meta analysis for 6 studies was 1.3 with CI 1.0, 1.6	Funded by Monsanto. Study discussion said the slight significance could be explained by individual studies being under powered, exposure misclassification, selection bias due to differential enrollment and follow up, poor adjustment for confounding
Author(s)	Study	Design	Exposure	Disease	Covariates	Result	Notes
[16] Sorahan, 2015	Multiple Myeloma and Glyphosate Use: A Re-Analysis of US Agricultural Health Study (AHS) Data	Re-analysis of a prospective cohort study	Glyphosate	Multiple Myeloma	Age, gender, smoking history, cigarette smoking, use of alcohol, family history of cancer, level of education, use of ten other pesticides	RR of 1.12 (95% CI 0.50 to 2.49) for ever-use of glyphosate. Additional adjustment for lifestyle factors and use of ten other pesticides had little effect (RR 1.24, 95% CI 0.52 to 2.94).	No significant correlation between glyphosate use and multiple myeloma

Some studies examined DNA damage, a potential intermediary to cancer, and its links to glyphosate exposure. Though DNA can repair itself, DNA repair mechanisms that are ineffective or error-prone may perpetuate mutations. This is a major way by which DNA damage, caused by radiation or chemical carcinogens, induces tumor formation. Thus, cellular DNA-repair processes have been implicated both in protecting against and contributing to the development of cancer [17]. DNA Damage is measured by comet assay (single-cell gel electrophoresis), a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks [18].

In-vitro studies have documented DNA damage from glyphosate and its metabolite, AMPA (Table 2). All studies found significant levels of DNA damage from glyphosate and its metabolite, AMPA. These studies represent biological plausibility that glyphosate and AMPA lead to DNA damage, but do not test exposure levels that might occur under natural conditions of glyphosate use, such as human exposure through inhalation.

Table 2: Experimental in-vitro human cell studies with glyphosate as exposure and DNA damage as outcome							
Author(s)	Study	Design	Exposure	Disease	Covariates	Results	Notes
[19] Koller, VJ *et al* 2012	Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells.	Experimental	Glyphosate	DNA in buccal epithelial cell line	n/a	DNA migration occurred in single cell electrophoresis assays at doses greater than 20 mg/L	This study noted that comparisons with lymphocytes and cells from other organs indicate that epithelial cells are more susceptible to DNA damage from glyphosate and Round Up and based on the methodology on exposure via inhalation in the field.

Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[20] Townsend M, *et al* 2017	Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on toxicity	Experimental	Glyphosate, various concentrations	DNA damage quantified by comet assay	n/a	Glyphosate is lethal to Raji cells greater than 10 mM and no cytotoxic effects below 100 µM. Concentrations of 1 mM and 5 mM induce statistically significant DNA damage following 30-60 minutes of treatment, with cells fully repaired after 60 minutes.

Table 2 (cont'd):						
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[21] F. Mañas, *et al* 2009	Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the comet assay and cytogenetic tests	Experimental	AMPA (metabolite of glyphosate) in	DNA damage assessed by comet assay	n/a	P<0.05 "In human lymphocytes we found statistically significant clastogenic effect AMPA at 1.8 mM compared with the control group.

Two animal studies reported significant levels of DNA damage in mice 24 hours after in vivo exposure to glyphosate (Table 3). These studies used injection as the route of exposure and therefore dosing amounts cannot be directly compared to those from human studies. However, when considered with the in-vitro human cell studies in Table 2, these experimental in-vitro and animal studies showing DNA damage resulting from glyphosate exposure motivate studies of DNA damage from glyphosate exposure in occupational settings.

Table 3: Experimental in vivo animal studies with glyphosate as the exposure and DNA damage as the outcome						
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[22] Bolognesi *et al*, 1997	Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup	Experimental	RoundUP injected intraperitoneally in mice as 300 mg/kg bw	DNA Damage of liver	n/a	P<0.05 after 24 hr of a single dose
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[23] F. Mañas, *et al* 2009	Genotoxicity of glyphosate, the environmental metabolite of glyphosate, assessed by the comet assay and cytogenetic tests	Experimental	Glyphosate at 200 mg/kg bw	DNA damage assessed by comet assay	n/a	P< 0.01 at the lowest dose after 24 hours. "in vivo genotoxicity was evaluated through the micronucleus test in mice. In the Comet assay, the level of DNA damage in exposed cells at 2.5–7.5 mM showed a significant increase compared with the control group".

Investigators also have explored DNA adducts, another possible intermediary of cancer, and its relation to glyphosate exposure. A DNA adduct is a piece of DNA covalently bonded to a chemical. When a chemical binds to DNA, the DNA becomes damaged, thereby increasing the opportunity for abnormal replication. A higher DNA adduct burden is associated with a greater cancer risk, perhaps alone or when accompanied by additional factors such as infections, inflammation, or impairment of DNA repair [24]. A 2012 systematic review by M. Poirier reviewed twelve studies and concluded that specific types of DNA adducts measured in humans contribute a relatively modest (1.6- to 9.1-fold) increase in human cancer risk [24]. At least three studies have reported significantly greater levels of DNA adducts in participants' white blood cells sampled after periods of high pesticide exposure compared to cells sampled after low periods of exposure (Table 4). Thus, DNA adducts may be a useful measure for assessing glyphosate's impact on DNA in occupational settings.

Table 4: Environmental studies with various pesticides as exposures and DNA adducts as outcome						
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[25] J. Le Goff *et al.* 2005	Seasonal variations of DNA-adduct patterns in open field farmers handling pesticides	Case-crossover	Various insecticides, fungicides, and pesticides	DNA adducts	Age, tobacco use	In the farmer group, relative adduct level increased by a factor of 3.4 between the heavy pesticide use sampling period from May-June and the low use period of January.
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[26] Antonio Gómez-Martín *et al*	Increased N7-Methyldeoxyguanosine DNA adducts after occupational exposure to pesticides and influence of genetic polymorphisms of paraoxonase-1 and glutathione S-transferase M1 and T1.	Case-crossover	Agricultural pesticides inside greenhouses	DNA adducts	Age, gender, height, weight, smoking history, alcohol, coffee, and tea intake, use of PPE	Statistically significant DNA adducts from low exposure to high exposure sampling periods, however in this study it is not possible to link the observed increased to a particular pesticide.

Table 4 (cont'd):						
Author(s)	Study	Design	Exposure	Disease	Covariates	Results
[27] Jerome Gallois, *et al*.	DNA Adduct variations in non-smoking crop farmers: Potential relationship with occupational exposure to pesticides?	Prospective cohort	Various insecticides, herbicides, fungicides	DNA adducts	Age, medication, welding activities	Statistically significant DNA adducts measured between April-July from randomly sampled time periods over a three-year study period.

Orchard Workers' Exposure to Glyphosate

Orchard employees are an under-studied occupational group with respect to pesticide exposures. While epidemiological studies exist examining DNA damage and DNA adducts in other glyphosate-exposed agricultural workers, currently no studies are publicly available that specifically study orchard workers, glyphosate exposure, and DNA adducts.

Northwest Michigan, with its abundant orchards and orchard workers, may be an ideal location to examine effects of glyphosate exposure on rates of DNA damage. According to the United States Department of National Agriculture Statistics Service (NASS), in 2015, 77% of Fruit and Tree Nut Operations in Michigan used pesticides for weed control [28]. This database does not denote type of pesticide, but as of 2014, glyphosate accounted for 90% of agricultural herbicide use [2]. In 2012 in Michigan (the latest year available for this data), there were 1,265 orchard operations with one operator, and 1,237 operations with at least two operators [29].

An anecdotal account from a Northern Michigan orchard operator stated that glyphosate (trade name RoundUp) is applied once a season, and is the sole herbicide applied. Standard personal protective equipment of white suits and gloves is recommended, but they are rarely worn. The concentration is about one quart of RoundUp per 100 gallons of water. The spraying boom (spraying arm) is covered to prevent the wind from blowing it away from the ground [20].

Measuring Glyphosate Exposure

Glyphosate exposure can be measured by measuring glyphosate and its metabolite, aminomethylphosphonic acid (AMPA) in urine, as described in the results of the following two studies: Y. Hory *et al* determined that high ratios of glyphosate to AMPA were detected in a human patient's serum 8 hrs (22.6 µg/mL glyphosate to 0.18 µg/mL AMPA) and 16 hrs (4.4 µg/mL glyphosate to 0.03 µg/mL AMPA) post-ingestion, as well as in the patient's urine. This indicates that glyphosate metabolism was minimal [31]. Acquavella, J., *et al* analyzed data from the Farm Family Exposure Study. Researchers collected urine samples from farm families in South Carolina and Minnesota. On the day of application, 60% of farmers had a detectable level of glyphosate in their urine of at least 1 ppb. The geometric mean of glyphosate detected was 3 ppb, with a maximum value of 233 ppb. Mean urinary concentrations of glyphosate were higher in farmers who did not use rubber gloves during application [32].

Summary

The IARC Working Group concluded that though there is limited evidence of glyphosate's carcinogenicity in humans, there is sufficient evidence of its carcinogenicity in experimental animals, and therefore they reclassified glyphosate as a 'probable carcinogen' [9]. However, animal studies of pesticide carcinogenicity often use doses and routes of exposure that are not representative of exposures in humans. The limitations in human studies stem, in part, from the long and difficult task of gathering enough cases of rare outcomes such as cancer in prospective studies and the challenges of obtaining accurate pesticide exposure data through retrospective case-control studies. To address these challenges, some investigators resort to using intermediaries of cancer risk, such as DNA damage and DNA adducts, which occur more often than cancer and appear soon after the carcinogen exposure period. This approach could be useful in exploring links between occupational exposure to glyphosate and future cancer risk, but to our knowledge it has not been applied to orchard workers. We will propose a study that measures levels of DNA adducts in orchard workers pre and post glyphosate exposure. To improve accuracy of glyphosate exposure levels, we will assess urinary levels of the glyphosate metabolite, AMPA, and glyphosate as well as self-reported use of personal protection equipment. In addition, we plan to consider potential effect modifiers such as demographics, lifestyle and health history. The orchard industries in Northwest Michigan provide a unique opportunity to include an understudied population in estimating glyphosate's potential impact on cancer risk.

R03: Specific Aims

1. Conduct a longitudinal study that compares prevalence of DNA adducts pre-and post-glyphosate exposure (individuals serve as their own control) among orchard employees. DNA adducts will be assessed from plasma samples and compared to levels of AMPA, the metabolite of glyphosate, in urine. Both urine and blood will be collected within 24 hours before and within 24 hours after the first exposure of the season applications.

Hypothesis 1: Evidence of DNA adducts will increase in the period following glyphosate exposure as compared to the period prior to glyphosate exposure.

2. Measure applicators' adherence to personal protection guidelines and determine if adherence reduces glyphosate exposure levels (lower urine AMPA) and thereby limits any increase in DNA adducts.

Hypothesis 2: Greater adherence to personal protection guidelines will result in decreased glyphosate exposure and less increase in DNA adducts from pre to post exposure.

Research Strategy Significance

Glyphosate, also known by the trade name Roundup, is a broad-spectrum herbicide widely used in the United States [1]. Glyphosate kills plants and bacteria by inhibiting the bacterial and plant enzyme enolpyruvylshikimate-phosphate synthase (EPSPS) [1]. The company Monsanto, the maker of Roundup, has developed a way to introduce a bacterial gene for a glyphosate-resistant EPSPS into plants so glyphosate can be used for weed control on otherwise glyphosate-susceptible crops [1].

As of 2014, glyphosate accounted for 90% of U.S. agricultural herbicide use. Glyphosate was introduced in 1974 and was not widely used until 1996 when genetically engineered soybeans, corn, and cotton were developed to resist glyphosate [2]. Glyphosate use increased from 12.5 million pounds in 1995 to 250 million pounds in 2014, a 20-fold increase [2]. According to the United States Department of National Agriculture Statistics Service (NASS), in 2015, 77% of Fruit and Tree Nut Operations in Michigan used pesticides for weed control [28]. In 2012 in Michigan (the most recent year available for this data), there were 1,265 orchard operations with one operator, and 1,237 operations with at least two operators [29]. Dr. Nikki Rothwell, a Michigan State University Extension horticulturist, confirmed that glyphosate is used in orchards Northwest Michigan [33].

Glyphosate is in a class of chemical substances known as organophosphates. Exposure to organophosphates has been associated with disruption of acetylcholinesterase (AChE) in the central and peripheral nervous system. AChE inhibitors can be divided into two groups: temporary (therapeutic uses in pharmacology) and irreversible (such as from organophosphorus compound exposure) [3]. Cellular DNA-repair processes have been implicated both in protecting against and contributing to the development of cancer [5]. Oxidative stress from AChE reduction may lead to genotoxicity from both acute and chronic exposures. Oxidative stress is the result of an imbalance between reactive oxygen species (free radicals) and the antioxidant defense system. Organophosphate-induced free radicals attack lipids, proteins, and DNA resulting in single and double strand breaks [4]. Though DNA can repair itself, DNA repair mechanisms that are ineffective or error-prone may perpetuate

mutations which is one way DNA damage caused by radiation or chemical carcinogens is known to induce tumor formation [17].

In August 2015, The International Agency for Research of Cancer (IARC), under the World Health Organization (WHO), published an updated monograph on glyphosate and their rationale for upgrading their classification of glyphosate from possibly carcinogenic to humans (Group 2B) to probably carcinogenic to humans (Group 2A) [8, 10].

The 2016 WHO monograph examined cohort, case-control, and experimental in vivo animal and in vitro human glyphosate exposure studies. Exposure was defined as ever personally mixing or applying products containing glyphosate and was quantified by cumulative lifetime days of use. Glyphosate was significantly associated with some specific cancers, but not with cancers analyzed as a group (combined melanoma, multiple myeloma, Non-Hodgkins Lymphoma, leukemia, and cancers of the oral cavity, colon, rectum, pancreas, kidney, bladder, and prostate) [10].

Results from cohort and case control studies of glyphosate exposure and risk of Non-Hodgkins Lymphoma (NHL) have been inconsistent. One case-control study noted a significant association with an odds ratio (OR) of slightly over three [12], whereas a pooled analysis of three case-control studies found a slightly significant association with an OR of 1.3 [13]. A prospective cohort study found no significant association between glyphosate and NHL [16]. A systematic review and meta-analysis, funded by Monsanto, combined six studies and reported an OR of 1.3 for glyphosate exposure and NHL [15].

Glyphosate and its metabolite, aminomethylphosphonic acid (AMPA), show the potential to induce genotoxicity in studies of human cells in vitro and in vivo, and in laboratory

animal studies with exposure through ingestion or injection [10]. Epidemiological studies assessing community-level glyphosate exposure observed increased DNA damage two weeks to two months after aerial spraying [10]. In three separate in vitro studies of human cells, investigators demonstrated significant increases in levels of DNA damage following exposure to glyphosate [19, 20, 21]. Two case-control and one prospective cohort study determined statistically significant increases in levels of DNA adducts from samples taken during periods of high pesticide use versus low pesticide use [25, 26, 27].

Occupational exposures to glyphosate among agricultural workers may be of concern, particularly in circumstances of faulty spraying equipment, accidental spills, not following standards requiring use of personal protective equipment (PPE), and use during windy conditions [5]. Personal protection guidelines for Roundup PRO and Roundup PROMAX include use of long pants and shirts, closed-toe shoes, gloves and eye protection, but do not require or recommend breathing protection [6]. The Environmental Protection Agency Worker Protection Standard does require the use of respirators, but owners of agricultural establishments and immediate family members are exempt from complying with the standard [7]. Personal protective equipment (PPE) guidelines are often are ignored. Given the uncertainties of health effects following glyphosate exposure, occupational groups involved in glyphosate spraying would benefit from information about: 1) factors that contribute to individual-level exposure to glyphosate; and 2) the cellular-level effects of glyphosate exposure. The latter may serve as a motivating element to minimize exposure through maximum adherence to current PPE guidelines, or the adherence to the EPA Worker Protection Standard.

Innovation

The 2015 glyphosate monograph does not include information on DNA adducts induced from occupational exposures. The Working Group concluded that limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals was the rationale behind the reclassification of glyphosate to 'probably carcinogenic to humans' [10]. However, gaps in knowledge remain because the routes of exposure routinely used in animal models, e.g. injection, do not match those of typical occupational exposure routes, and there are a limited number of occupational epidemiological studies. To assess glyphosate's potential for carcinogenicity, without waiting years for a prospective cohort study of cancer in orchard sprayers, this proposed workplace exposure study examines a frequently used carcinogenic intermediate, i.e. DNA adducts. A DNA adduct is a piece of DNA covalently bonded to a chemical; this adduct can lead to abnormal cell replication and in turn serve as a precursor to cancer. The effects of DNA adducts may be compounded by additional factors such as certain infections, other causes of inflammation, or impaired DNA repair [24]. Though no specific studies were found that examine glyphosate or AMPA induced DNA adducts and cancer outcomes, a 2012 systematic review examined twelve investigations showing 2- to 9-fold increased Relative Risks (RR) or Odds Ratios (OR) for cancer in the 25% of individuals having the highest DNA adduct levels, compared to the 25% of matched individuals with the lowest DNA adducts [24]. This review does not include correlations with DNA adducts and Non-Hodgkin Lymphoma, but does find relative risks and/or odds ratios of at least 2.8 in breast, liver, stomach, colon, bladder, and lung cancers. Together, the data from these studies suggest

that a reduction in human DNA adduct level is likely to produce a reduction in human cancer risk.

This proposed small-scale study focused on glyphosate exposures in agricultural workers has several innovative components that can inform methodology needed for a larger study. The focus of this proposed work is to evaluate exposure to glyphosate in workers involved in spraying fruit, a major farm product of Michigan. We use a pre and post spraying design to assess: 1) individual glyphosate exposure by measuring one of its metabolites, AMPA, detected in urine; and 2) DNA adducts in white blood cells. The study also considers factors that might modify a relationship between glyphosate spraying and DNA adducts, e.g. adherence to PPE, lifestyle, and health history. Before launching a larger, definitive study, the innovative methods proposed here need testing on a smaller group in the field. Results from this smaller study will generate estimates of glyphosate exposure levels in fruit tree sprayers, and effect size (correlation between AMPA levels and levels of DNA adducts) to guide sample size determinations for the larger study. This study also can identify additional covariates that affect individual levels of glyphosate metabolite, and therefore would need to be incorporated in future studies. The orchard industry in Northwest Michigan provides a unique opportunity to include an understudied population and closely examine opportunities to reduce glyphosate exposure and its impact.

Approach

The research team will collaborate with the Michigan Department of Agriculture and Rural Development, the Northwest Michigan MSU Extension Office and Northwest Michigan Community Action Centers (for migrant workers) to reach out to commercial applicator teams and owners of privately owned orchards (whom typically apply their own pesticides). A Spanish-English translator will be available for Spanish speaking participants.

Methods

Study Sample and Design:

This study will enroll 20 glyphosate applicators, ten from corporate orchards and ten from privately owned and operated orchards. The two main orchard products in Michigan [34] are apples and cherries; we will try to have both of these orchard types represented in our study. Enrollment will begin in February of the study year, in preparation for the spraying season. This longitudinal study will compare the prevalence of DNA adducts pre-and post-glyphosate exposure (individuals serve as their own control) among orchard applicators.

Eligibility and Exclusion Criteria and Recruitment:

An initial questionnaire will be used to determine eligibility based on inclusion and exclusion criteria. Eligible individuals are those who plan to work as an applicator of glyphosate during the year of the study in Michigan orchards. Exclusion criteria are history of organ transplantation, human immunodeficiency virus infection and/or current cancer diagnosis.

Eligible applicators will be randomly selected to participate in this study. Study staff will describe details of the study and obtain participant consent along with contact information for the purpose of regular follow-up (2/month) by study staff. Through these contacts study staff will learn about participants' scheduling of first glyphosate spraying in the season.

Data Collection:

Approximately 24 hours *before* glyphosate spraying, study staff trained in phlebotomy will meet with the applicator to: 1) administer a baseline questionnaire that asks about potentially relevant covariates such as history of pesticides applied, age, gender, recent smoking and alcohol use, and welding activities in the past year, and typical use of PPE; 2) collect a urine sample; and 3) collect a blood sample**.** Urine will be collected in 500 mL high-density polyethylene wide mouth containers and blood will be collected in EDTA Vacutainer tubes.

Approximately 24 hours *after* glyphosate spraying, the same study staff will meet with applicator to: 1) administer a follow-up questionnaire that asks about exact time of spraying, duration of spraying time, PPE used, and levels of alcohol and smoking in the interval between first sample collection and second sample collection. The PPE questions will be based off of the Agricultural Health Survey's questionnaire regarding PPE [35], and; 2) collect a urine sample; and 3) collect a blood sample.

Exact times of sample collection and spraying will be recorded. All biological samples will be placed in dry ice immediately after collection and promptly transported to the study laboratory for freezer storage. Laboratory personnel analyzing AMPA levels and DNA adducts

will be blinded to timing of sample collection, i.e. before or after exposure. Following collection of pre and post exposure information and biological samples, all identifying information will be eliminated and information will only be linked by an ID number, not traceable to an individual.

Exposure Measure:

AMPA, the metabolite of glyphosate, will be measured in pre and post exposure urine samples as an indication of glyphosate exposure levels. AMPA specimen collection will be within 24 hours of exposure based on a previous study that detected AMPA 24 hours after glyphosate exposure but not by 3 days post-exposure [32]. Samples will be aliquoted and frozen in a -40 degree Celsius freezer and analyzed in a CLIA certified lab using creatinine to measure urine concentration and liquid-chromatography-mass spectrometry to measure AMPA concentration.

Outcome Measure:

DNA adducts will be evaluated in blood cells collected within 24 hours before and within 24 hours after glyphosate exposure, thereby minimizing effects of individual-based DNA repair time. Experimental in-vivo studies have shown statistically significant DNA damage 24 hours after glyphosate exposure in mice [22, 23]. The 24-hour pre and post exposure sample collection protocol is also efficient because it allows research staff to collect blood and urine at the same time. Samples will be centrifuged to separate plasma from erythrocytes. Plasma will be aliquoted and frozen in a -40 degree Celsius laboratory freezer. DNA from white blood cells in plasma will be processed using the PureGene kit to isolate DNA and assayed using the P -

postlabeling assay due to its high sensitivity and the low study sample size [36]. Following the process in Gallois et al, the DNA adducts will be separated with thin layer chromatography, and given a radioactive labeling indicator. Levels of radioactivity in the adducts will be read with a scintillator and will be measured against the standard control to determine the relative adduct level (RAL) [37].

Statistical Analysis:

Means and percentages will be used to describe study participant demographics of age, gender, smoking and alcohol use, welding activity, and PPE use.

Aim 1: Participants will serve as their own controls. AMPA levels and DNA adduct levels before and after exposure will be analyzed as a paired t-test using a two-sided alpha = 0.05. AMPA levels will be measured in parts-per-billion and DNA adducts will be measured by determining the RAL, described above. The RAL is not normally distributed and will need to be log-transformed. Based on Gallois et al, 40% of our samples may have non-quantifiable RALs, which will however be an indicator for the sample size needed in a larger R01.

The DNA samples will be assayed twice and the mean RAL for each participant will be compared to the AMPA ppb in urine. Relationships between changes in AMPA concentrations and changes in DNA adducts, along with time varying covariates that could serve as confounders (e.g. smoking , alcohol use) will be evaluated in regression models that account for clustering within individual, e.g. generalized estimating equation.

Aim 2: Analyses in Aim 1 will be repeated after stratifying applicators by: 1) spraying on cherry vs apple orchard on test day; 2) corporate-owned versus privately owned orchard; and 3) reported level of PPE use, e.g. divided at the median level. The small sample sizes generated in these stratified models may preclude firm conclusions but can be used to motivate the design of a larger study. Points will be assigned to each PPE used, and PPE will be stratified into non-porous gloves, surgical/cloth mask, respirator, long shirts and pants, and liquid-resistant boots. While this small study does not have adequate statistical power to detect moderate levels of effect modification, these analyses will inform plans for larger studies.

Strengths and Limitations:

The strengths of this study include the use of a biomarker (AMPA) to measure glyphosate exposure and the assessment of PPE per regulatory standards and its relation to exposure levels. Laboratory personnel analyzing AMPA levels and DNA adducts will be blinded to timing of sample collection, i.e. before or after exposure to avoid bias. Study subjects serving as their own controls increases the study's power while using fewer participants and reduces the chance of confounding.

Limitations of this study include the possibility that participants will not accurately report their adherence to personal protection guidelines during the study period (misclassification of effect modifier), or will alter their adherence behaviors just during the study period (exposure levels won't represent usual exposure when not in study). This will be minimized to every extent possible by ensuring confidentiality, explaining the importance of the study to participants and the use of study staff to interview participants. In addition, the small

sample size of this study will result in wide confidence intervals around effect size estimates; but these estimates are necessary for planning a large-scale study.

If the hypotheses of this R03 are supported by the results, this study would motivate a larger study to examine relations between glyphosate exposure and DNA adducts, a precursor for cancer, in glyphosate sprayers working in orchards. Results of this study may also provide evidence of potential risk reduction of DNA adducts with PPE use and be used as an educational resource for PPE use in the industry, as well as support the need for continued scientific study to examine the effectiveness of regulatory standards aimed at worker protection.

BIBLIOGRAPHY

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1. Wiersema R., et al Glyphosate Degradation Pathway [Internet]. Eawag-bbd.ethz.ch. 2018 [cited 26 September 2018]. Available from: http://eawag-bbd.ethz.ch/gly/gly_map.html

2. Benbrook C., et al. Trends in glyphosate herbicide use in the United States and globally [Internet]. Springer Open. 2016 [cited 10 September 2018]. Available from: http://enveurope.springeropen.com/articles/10.1186/s12302-016-0070-0

3. How V. et al. Biological monitoring of genotoxicity to organophoshate pesticide exposure among rice farmers: Exposure-effect continuum study. Journal of Occupational Health and Epidemiology [Internet]. 2014 [cited 11 December 2016];. Available from: https://www.researchgate.net/publication/265743191_Biological_monitoring_of_genotoxicity_to_organophoshate_pesticide_exposure_among_rice_farmers_Exposure-effect_continuum_study.

4. Čolović M. , et a. lAcetylcholinesterase Inhibitors: Pharmacology and Toxicology. Current Neuropharmacology [Internet]. 2013 [cited 11 December 2016];11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3648782/.

5. Damalas C, Eleftherohorinos I. Pesticide Exposure, Safety Issues, and Risk Assessment Indicators. International Journal for Environmental Research and Public Health [Internet]. 2011 [cited 21 August 2018]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3108117/.

6. Roundup ProMax Label [Internet]. 1st ed. [cited 21 August 2018]. Available from: http://www.domyownpestcontrol.com/msds/roundup_PromaxLabel.pdf

7. Requirements under the Worker Protection Standard for Owners of Agricultural Establishments and Immediate Family | US EPA [Internet]. US EPA. 2015 [cited 12 February 2020]. Available from: https://www.epa.gov/pesticide-worker-safety/requirements-under-worker-protection-standard-owners-agricultural

8. Glyphosate. Some Organophosphate Insecticides and Herbicides [Internet]. 2016 [cited 8 August 2018];112. Available from: http://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Evaluation-Of-Carcinogenic-Risks-To-Humans/Some-Organophosphate-Insecticides-And-Herbicides-2017

9. IARC Monographs- Classifications [Internet]. Monographs.iarc.fr. 2017 [cited 8 August 2018]. Available from: http://monographs.iarc.fr/ENG/Classification/

10. Glyphosate [Internet]. World Health Organization; 2015 [cited 16 August 2018]. Available from: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono112-10.pdf

11. Fritschi L., et al. Occupational Exposure to Pesticides and Risk of Non-Hodgkin's Lymphoma [Internet]. Oxford Journals American Journal of Epidemiology. 2016 [cited 5 August 2018]. Available from: http://aje.oxfordjournals.org/content/162/9/849.full

12. De Roos A., et al. Cancer Incidence among Glyphosate-Exposed Pesticide Applicators in the Agricultural Health Study. Environmental Health Perspectives [Internet]. 2016 [cited 5 August 2018]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1253709/?tool=pmcentrez

13. De Roos A., et al. Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men [Internet]. BMJ. 2016 [cited 5 August 2018]. Available from: http://oem.bmj.com/content/60/9/e11.full.

14. Hardell L., et al Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: pooled analysis of two Swedish case-control studies. Taylor and Francis Online [Internet]. 2002 [cited 5 August 2018];43(5). Available from: http://www.tandfonline.com.proxy2.cl.msu.edu/doi/abs/10.1080/10428190290021560

15. Chang E, Delzell E. Systematic review and meta-analysis of glyphosate exposure and risk of lymphohematopoietic cancers. Journal of Environmental Science and Health, Part B [Internet]. 2016 [cited 8 August 2018];51(6):402-434. Available from: http://www.tandfonline.com.proxy1.cl.msu.edu/doi/abs/10.1080/03601234.2016.1142748

16. Sorahan T. Multiple Myeloma and Glyphosate Use: A Re-Analysis of US Agricultural Health Study (AHS) Data. International Journal of Environmental Research and Public Health [Internet]. 2015 [cited 10 March 2018];12(2):1548-1559. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4344679/

17. Lodish H, Berk A, Zipursky S. DNA Damage and Repair and Their Role in Carcinogenesis [Internet]. Ncbi.nlm.nih.gov. 2000 [cited 2 October 2018]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21554/

18. Collins A. The Comet Assay for DNA Damage and Repair: Principles, Applications, and Limitations [Internet]. PubMed. 2004 [cited 1 October 2018]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/15004294

19. Koller V, Fürhacker M, Nersesyan A, Mišík M, Eisenbauer M, Knasmueller S. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. Archives of Toxicology [Internet]. 2012 [cited 8 August 2018];86(5):805-813. Available from: https://search-proquest-com.proxy1.cl.msu.edu/docview/1008948592?pq-origsite=summon’

20. Townsend M, Peck C, Meng W, Heaton M, Robison R, O'Neill K. Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity. Regulatory Toxicology and Pharmacology [Internet]. 2017 [cited 31 July 2017];85:79-85. Available from: http://www.sciencedirect.com/science/article/pii/S0273230017300235?via%3Dihub

21. Mañas F, Peralta L, Raviolo J., et al Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests. Ecotoxicology and Environmental Safety [Internet]. 2009 [cited 10 March 2018];72(3):834-837. Available from: https://www.sciencedirect.com/science/article/pii/S0147651308002662

22. Bolognesi, C., et al (1997). Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup. [online] Available at: https://www.researchgate.net/publication/231555170_Genotoxic_Activity_of_Glyphosate_and_Its_Technical_Formulation_Roundup [cited 16 August 2018].

23. Mañas F, Peralta L, Raviolo J., et al. Genotoxicity of glyphosate, the environmental metabolite of glyphosate, assessed by the comet assay and cytogenetic tests. [Internet]. 2009 [cited 16 August 2018];. Available from: https://www.sciencedirect.com/science/article/pii/S1382668909000258

24. Poirier M. Chemical-induced DNA Damage and Human Cancer Risk [Internet]. Discoverymedicine.com. 2018 [cited 13 October 2018]. Available from: http://www.discoverymedicine.com/Miriam-C-Poirier/2012/10/29/chemical-induced-dna-damage-and-human-cancer-risk/

25. Le Goff J, André V, Lebailly P, Pottier D, Périn F, Périn O et al. Seasonal variations of DNA-adduct patterns in open field farmers handling pesticides. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2005;587(1-2):90-102. [cited 20 April 2020].

26. Gómez-Martín A, Altakroni B, Lozano-Paniagua D, Margison G, de Vocht F, Povey A et al. Increased N7-methyldeoxyguanosine DNA adducts after occupational exposure to pesticides and influence of genetic polymorphisms of paraoxonase-1 and glutathioneS-transferase M1 and T1. Environmental and Molecular Mutagenesis. 2014;56(5):437-445. [cited 20 April 2020].

27. Gallois J, Pottier D, Houssin M, Le Goff J, André V. DNA adduct variations in non-smoking crop farmers: Potential relationship with occupational exposure to pesticides? Environmental Toxicology and Pharmacology. 2011;32(1):1-9. [cited 20 April 2020].

28. USDA/NASS QuickStats Ad-hoc Query Tool [Internet]. Quickstats.nass.usda.gov. 2018 [cited 20 August 2018]. Available from: https://quickstats.nass.usda.gov/results/2C111BCB-7DE8-3A84-BB5F-8073D7E9CF25

29. USDA/NASS QuickStats Ad-hoc Query Tool [Internet]. Quickstats.nass.usda.gov. 2018 [cited 20 August 2018]. Available from: https://quickstats.nass.usda.gov/results/9D6BD338-2DF4-305E-B21C-36EC7CA54969

30. Swygert K. Round Up use in orchards. 2018.

31. Hori Y. et al. Determination of the herbicide glyphosate and its metabolite in biological specimens by gas chromatography-mass spectrometry. [Internet]. Ncbi.nlm.nih.gov. 2003 [cited 21 August 2018]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/12731658

32. John F Acquavella M., et al. Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. [Internet]. PubMed Central (PMC). 2004 [cited 21 August 2018]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1241861/

33. Rothwell, N. Herbicide use in Northwest Michigan orchards. 2018.

34. USDA/NASS 2019 State Agriculture Overview for Michigan [Internet]. Nass.usda.gov. 2020 [cited 20 August 2020]. Available from: https://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=MICHIGAN

35. Questionnaires & Study Data | Agricultural Health Study [Internet]. Aghealth.nih.gov. 2020 [cited 24 July 2020]. Available from: https://www.aghealth.nih.gov/collaboration/questionnaires.html

36. Phillips D. Methods of DNA Adduct Determination and Their Application to Testing Compounds for Genotoxicity. Environmental and Molecular Mutagenesis 35:222–233 [Internet]. 2000 [cited 20 July 2020];35(222-233). Available from: https://superfund.berkeley.edu/pdf/320.pdf

37. Gallois J, Pottier D, Houssin M, Le Goff J, André V. DNA adduct variations in non-smoking crop farmers: Potential relationship with occupational exposure to pesticides. Environmental Toxicology and Pharmacology. 2011;32(1):1-9. [cited 20 April 2020].