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GENOTOXIC EFFECTS AMONG BOLIVIAN FARMERS EXPOSED TO MIXTURES OF PESTICIDES: POPULATION AND IN VITRO BASED STUDIES

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Genotoxic effects among Bolivian farmers exposed to mixtures of pesticides: population and in vitro based studies

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ABSTRACT

Human exposure to pesticides has increased exponentially in recent decades, especially in low- and middle-income countries where regulations on the use of pesticides and personal protective equipment (PPE) are not fully controlled. Studies have shown that compared to the general population, people occupationally exposed to pesticides have a higher risk of developing acute and chronic adverse health effects, and increased risk of genotoxic damage and cancer. The general objective of this thesis was to evaluate the correlation between exposure to mixtures of pesticides and genotoxicity in the agricultural Bolivian population. For this, a cross-sectional study was used in three agricultural communities, whose production represents almost the entire diversity of the country. The use and exposure to pesticides were determined by applying a survey on lifestyle factors, behaviors, and pesticide management, and by analyzing 10 urine pesticide metabolites (UPM). Our results demonstrated that the Bolivian agricultural population is highly exposed to mixtures of pesticides. High exposure levels of chlorpyrifos, 2,4-D, and some pyrethroids were found, and especially among men. Furthermore, we found that farmers who were better at following directions for using pesticides and PPE, in general, were less exposed to pesticides (Paper I). We also investigated the correlation between pesticide exposure and genotoxic effects. We found that high exposure levels of certain pesticides, e.g. tebuconazole, 2,4-D, and cyfluthrin, was associated with high levels and increased risks of genotoxic damage (Paper II). To gain a better understanding of possible cellular effects of pesticide mixtures, we studied cytotoxicity and genotoxicity in human liver carcinoma cells (HepG2 cells) exposed to mixtures of pesticides, which were based on UPM and survey profiles. Our results showed that while neither of the mixtures nor their constituent pesticides induced formation of reactive oxygen species, increased levels of genotoxic damage were observed. Mixtures that were primarily composed of paraquat and cypermethrin demonstrated the highest genotoxic potency, as did paraquat and cypermethrin as single compounds. (Paper III). In conclusion, the results from our population and *in vitro* studies suggest that specific pesticides may act as drivers of toxic effects observed from exposure to mixtures. More studies are however necessary to get a clearer understanding of these effects. Finally, we want to emphasize the need to train farmers in pesticide management and personal protection to reduce exposure levels and thereby decrease the risk of health adverse effects.

RESUMEN

La exposición humana a plaguicidas ha aumentado exponencialmente en las últimas décadas, especialmente en países de bajos a medianos ingresos donde las regulaciones sobre el uso de plaguicidas y equipos de protección personal (PPE) no están completamente controlados. Estudios han demostrado que, en comparación con la población general, las personas expuestas ocupacionalmente a los plaguicidas presentan mayor riesgo de desarrollar efectos adversos a la salud, y un mayor riesgo a desarrollar daño genotóxico y cáncer. El objetivo general de esta tesis fue evaluar la correlación entre la exposición a mezclas de plaguicidas y la genotoxicidad en poblaciones agrícolas bolivianas. Se utilizó un estudio de corte transversal en pobladores de tres comunidades agrícolas, cuya producción representa casi toda la diversidad agrícola del país. El uso y la exposición a los plaguicidas se determinó aplicando una encuesta sobre estilo de vida, comportamientos y manejo de plaguicidas, y analizando 10 metabolitos de plaguicidas en la orina (UPM). Nuestros resultados demostraron que la población agrícola boliviana está altamente expuesta a mezclas de plaguicidas. Se encontraron altos niveles de exposición a clorpirifos, 2,4-D y algunos piretroides, especialmente en varones. Además, descubrimos que los agricultores que seguían las instrucciones para usar plaguicidas y PPE, estaban menos expuestos a los plaguicidas (Publicación I). Además, se investigó la correlación entre la exposición a plaguicidas y los efectos genotóxicos. Descubrimos que altos niveles de exposición a ciertos plaguicidas (tebuconazol, 2,4-D y ciflutrina) estaban asociados a altos niveles y mayor riesgo de presentar daño genotóxico (Publicación II). Por otra parte, para entender mejor los posibles efectos celulares de las mezclas de plaguicidas, se estudió la citotoxicidad y la genotoxicidad en células de carcinoma hepático humano (células HepG2) expuestas a nuestras mezclas de plaguicidas, basadas en UPM y perfiles de encuestas. Los resultados mostraron que, aunque ninguna de las mezclas ni sus plaguicidas constituyentes indujeron la formación de especies reactivas de oxígeno, si se observaron niveles aumentados de daño genotóxico. Las mezclas que estaban compuestas principalmente de paraquat y cipermetrina demostraron la mayor potencia genotóxica, al igual que cuando fueron evaluados individualmente (Publicación III). En conclusión, los resultados de nuestra población y los estudios *in vitro* sugieren que algunos plaguicidas pueden actuar como impulsores de los efectos tóxicos observados por la exposición a mezclas. Sin embargo, se necesitan realizar más estudios para comprender más claramente de estos efectos. Finalmente, queremos enfatizar la necesidad de capacitar a los agricultores en el manejo de plaguicidas e incentivar el uso de PPE para reducir los niveles de exposición y, de esa forma, disminuir el riesgo de efectos adversos a la salud.

LIST OF SCIENTIFIC PAPERS

I. **Barrón Cuenca J., Tirado N., Vikström M., Lindh C., Stenius U., Leander K., Berglund M., Dreij K.** "Pesticide exposure among Bolivian farmers: associations between worker protection and exposure biomarkers". Journal of Exposure Science & Environmental Epidemiology, 2019, https://doi.org/10.1038/s41370-019-0128-3.

II. **Barrón Cuenca J., Tirado N., Barral J., Ali I., Levi M., Stenius U., Berglund M., Dreij K.** "Increased levels of genotoxic damage in a Bolivian agricultural population exposed to mixtures of pesticides." Science of the Total Environment 695, 133942, 2019.

III. **Barrón Cuenca J., De Oliviera Galvão MF., Ünlü Endirlik B., Tirado N., Dreij K.** "*In vitro* cytotoxicity and genotoxicity of single and combined pesticides used by Bolivian farmers" (Manuscript).

CONTENTS

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
3-PBA	3-phenoxybenzoic acid
4F3PBA	4-fluoro-3-phenoxybenzoic acid
5-OH-TBZ	5-hydroxytiabendazole
8-OHdG	8-hydroxy-2-deoxyguanosine
AChE	Acetylcholinesterase activity
APP	Acute pesticide poisoning
CBMN	Cytokinesis-block micronucleus assay
CFCA	Chloro-3,3,3-trifluoro-1-propen-1-yl−2,2-dimethylcyclopropanecarboxylic acid
Com1	Community of Sapahaqui
Com2	Community of Villa Bolivar
Com3	Community of Villa 14 de Septiembre
DCCA	Cis/trans 3-(2,2-dichlorovinyl) −2,2-dimethylcyclopropane carboxylic acid
ECL	Enhanced chemiluminescence
ED	Endocrine disruptors
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
GHS	Globally Harmonized System
IARC	International Agency for Research on Cancer
IPCS	International Programme on Chemical Safety
IPM	Integrated Pest Management
LMICs	Low-middle income countries
LOD	Limit of detection
MCPA	4-chloro-2-methyl phenoxy acetic acid
MOA	Mode of action
OCPs	Organochlorine pesticides
OH-PYR	3-hydroxy-pyrimethanil

OPs	Organophosphates
PHI	Protection and handling index
PPE	Personal protective equipment
TCP	3,5,6-Trichloro-2-pyridinol
TEB-OH	Hydroxy-tebuconazole
UPMs	Urinary pesticide metabolites
US EPA	United States Environmental Protection Agency

1 INTRODUCTION

Agricultural production uses pesticides widely to prevent or reduce losses by pests, improving in some cases the quality of the product, and giving to the farmers a labor-saving, efficient and economical tool against the pest. Pesticides have been designed to kill and control certain organisms, and indeed, they create a risk of harm in people who use them [1].

During the last decade, the use of pesticides became very popular, especially in developing countries, to get into an international competitive agricultural market. However, the extensive use together with a lack of control by the authorities and the unconcern of the farmers has caused increased health risks. Additionally, the non-governmental environmental organization Greenpeace has identified different studies made in the general population showing detectable levels of pesticide metabolites in urine, indicating a possible indirect exposure through food and water contaminated with pesticides or by the air and dust in agricultural communities [2].

At present, the frequency of acute pesticide poisonings has increased among farmers and most likely due to the application of pesticides without previous training or knowledge of safety procedures, such as the use of personal protective equipment (PPE). Moreover, the routine of spraying pesticides during long periods of time exposes the farmers to chronic health problems such as diabetes or high blood pressure [3, 4]. On the other hand, long-term exposure to pesticides has been suggested to induce DNA damage and oxidative stress, increasing the risk of developing chronic diseases, or cancer in early adulthood [5, 6].

This thesis will focus on general concepts of exposure and health effects of pesticides and more specifically on the mechanisms involved in chronic health effects such as carcinogenesis. The use of biomarkers for determining exposure, effect, and susceptibility to pesticide exposure are also described.

1.1 PESTICIDES IN AGRICULTURE THROUGH THE HISTORY

Agriculture has been practiced for almost 10,000 years. Since ancient times, farmers have tried to find ways of controlling pests in order to avoid harvest loss. Sumerians used sulfur as pest control and other minerals such as mercury and arsenic were used by the Chinese. Greeks and Romans used mixtures of plants and some minerals for that effect. It is also known that smoke of different plants and some animal remains were used against mildew, blights, and insects. Pyrethrum, a natural insecticide made from the dried flower heads of chrysanthemum, has been

used for over 2000 years as a protection for stored grain, and a mixture of copper sulfate and lime called Bordeaux mixture, is still used as a fungus controller [7].

Arsenic–based pesticides were used for weeds and fungus control, but also in rice-killing operations during the Vietnam War [8]. In the 1940s, dichlorodiphenyltrichloroethane (DDT) was developed for insect control in crops, homes, and gardens as a controller of insect-borne diseases such as malaria. After their high persistence and environmental impact were demonstrated in the 1970s, the organochlorine pesticides were slowly replaced by other less persistent compounds like organophosphates (OPs) and carbamates (CBs) [7, 9]. However, since the 20th century, a large number of pesticides have been developed and their use has increased around 50-fold, especially in developing countries. As a result, regulatory agencies were created to control the use of pesticides to reduce the exposure population and contamination of the environment [10, 11]. From the 1990s, new pesticides with greater selectivity and better toxicological and environmental profiles were developed. However, in 1994, genetically modified crops (GM crops) were introduced to the market, designed to interact with their own pesticide [12]. Consequently, the concept of Integrated Pest Management (IPM) was introduced with the aim to reduce the use of pesticides in order to avoid mishandling and overuse of toxic pesticides through training in different pest-control techniques [13, 14].

1.2 PESTICIDE CLASSIFICATION

Pesticides can be classified based on their type of chemical e.g. organochlorines (OCPs), OPs, S-triazines, pyrethroids, etc., but also based on their target organism or targeted use as insecticides, herbicides, rodenticides, fungicides and so on. The World Health Organization (WHO) has also classified them by their health risk or hazard. This classification was based on a single or repeated exposure in a relatively short period of time, according to their oral or dermal toxicity. The classification goes from Ia (Extremely hazardous), Ib (Highly hazardous), II (Moderately hazardous), III (Slightly hazardous), and Unlikely to present a hazard in normal use [15]. The International Agency for Research on Cancer (IARC) has classified several pesticides according to their carcinogenic potential to humans [16, 17]. Another classification was made for the Globally Harmonized System, based on intrinsic properties of the pesticide, including physical, health and environmental hazards of the chemicals, along with some graphical communication such as pictograms, hazard statements, and the signal words “Danger” and “Warning” [18].

1.3 RISK ASSESSMENT AND HUMAN EXPOSURE TO PESTICIDES

1.3.1 Health risk assessment of pesticides

Since pesticides are extensively used worldwide in agriculture, they represent a significant risk of exposure to people occupationally exposed to them as well as the general population. Because of their hazardous properties and for being non-selective, even low levels can affect non-target organisms, especially susceptible populations [4]. In pesticide risk assessment, dose-relationships, exposure assessment, and potential health hazards must be identified. However, correlation (or association) does not always imply causation, therefore, many other possible causations (models) must be studied and eliminated before concluding causality. Nonetheless, the relationship between dose (magnitude of exposure) and the outcome incidence/severity can also help to prove causality [19]. To control several pests, pesticides are commonly applied as mixtures. Most of the risk assessment models were developed for single pesticide exposure, which might not be fully applicable for mixtures. For example, toxicokinetic interactions of one compound can alter the absorption, distribution, metabolism, or elimination of other compounds, making it difficult to know the cumulative effects of the exposure to a mixture of pesticides [20].

The Agency for Toxic Substances and Diseases Registry in the USA (ATSDR) have used physiologically based pharmacokinetic/toxicodynamic (PBPK/TD) models to assess the combined effects of mixtures. These models can predict internal doses levels and toxicokinetic parameters in different conditions for hypothetical exposures, and they can provide scientifically supportable results [21]. As an example, a PBTK/TD model was designed for assessing the interaction threshold for the combined toxicity of chlorpyrifos and parathion in rats. The results showed an additive interaction when the values were under the threshold and an antagonist effect when the values were above [22]. However, there are still many limitations for studying mixture effects by different pesticides in complex exposure scenarios, and ATSDR concluded than many more studies are needed for better understanding the toxicodynamic interactions of mixtures. To minimize that issue, the European Food Safety Authority (EFSA) in 2019 has published a guidance for using across their scientific committee when there is a necessity for evaluating the combined effects of chemical mixtures that potentially can be in food and feed [23]. Since the number of possible combinations of the mixtures can be infinite, the guidance works similarly as an evaluation of a single compound, and in the end, the risk can be quantified by comparing combined exposure and combined toxicity. This guidance also tries to estimate the overall risk adding up the doses for common effects and possible interactions, especially if the combined effect increases the toxicity [23].

1.3.2 Human exposure to pesticides

Pesticide exposure may occur in different ways, directly by the occupational activity and in domestic use, and indirectly through the consumption of food and water that contains remains of pesticides [24]. As with most chemicals, pesticides can enter the human body by skin absorption, ingestion, inhalation, and other routes of exposure. Moreover, the presentation, concentration, and formulation of pesticides are also important to consider [25].

The rate of absorption by the human body differs depending on the route of exposure. Human skin is considered the largest organ in the human body, and it has a great capability of absorption of substances such as pesticides [26]. This exposure may vary broadly depending on the amount of nude skin surface is in contact with the pesticide and the duration of the exposure. Sometimes other factors such as temperature, the humidity, and the lack of use of PPE can increase the skin absorption in people in direct contact [4, 27]. Oral exposure may occur for voluntary reasons or by accident due to carelessness, due to the reuse of empty bottles for storage of food or when the pesticides are transferred from their original bottle to a food container [28]. Additionally, drops of pesticides may reach the eye making a high local injury [29]. Moreover, its known that some pesticides have transplacental absorption properties that expose the fetus to high concentrations of pesticide metabolites [30, 31]. Many OCPs can accumulate in the adipose tissues. During the production of breast milk, the human body uses lipids from the adipose tissue, and accumulated OCPs in the adipose tissue can migrate to breast milk [32].

1.3.2.1 Populations exposed to pesticides

The worldwide and extensive use of pesticides makes almost all populations susceptible to exposure [33, 34]. Although exposure levels are highest in people who work in the manufacture of pesticides, exterminators of vector-diseases in public health, and farmworkers (occupational settings), the general population may also be exposed to low levels of pesticide mixtures throughout their lives. There are different sources of pesticide residues in the environment, such as in water supplies, fruits, and vegetables, or in the air that they breathe by living in the vicinity of areas where pesticides are applied [35]. To monitor the exposure levels for the general population health authorities have initiated surveillance programs. For example, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) started the French observatory for pesticide residues (ORP) in order to collect and analyze information on the presence of pesticides in different environments (e.g. phytosanitary products, biocides, etc.) for risk assessment purposes [36]. Moreover, the National Health and Nutrition Examination Survey (NHANES) led by the Center for Disease Control and Prevention (CDC)

in the USA collects information and biological samples, such as blood and urine, from the civilian population of all ages for biomonitoring proposes [37]. For example, a study used this database for evaluating urine concentrations of OP metabolites in relation to serum concentrations of testosterone and estradiol. They could detect OP metabolites in more than 50% of their samples and found a statistically significant inverse relationship between levels of the OP diethyl phosphate and testosterone [38].

1.3.2.2 Farmworkers

Agricultural activity is considered high risk for pesticide exposure. Farmworkers are highly exposed not only when they mix, load, transport, and apply pesticides, but also through accidental spills, leakages, or faulty spraying equipment. These factors may increase the frequency of pesticide use and the hours spending in the cultivation area during long periods of time and thus make them more vulnerable to develop chronic diseases [39, 40]. The use and type of pesticides can vary depending on the crops that the farmers are growing, the season, and the pest that they want to control or eliminate. The exposure could increase even more if the farmers mix many different pesticides at the same time for one application and if they do not follow the instructions on how to apply the pesticides, especially when they are unaware of safety guidelines on the use of PPE [21, 24]. Studies have demonstrated that farmers may forget fundamental sanitation practices such as taking a shower or washing hands after pesticide handling; therefore, family members of farmers may be exposed to pesticides through the take-home pathway [41]. In addition, agricultural work does not require that farmers are well educated, as a result, many farmers in low-to-middle income countries (LMICs) are illiterate or only have primary studies. This is of special concern for women, which increases the risk of not knowing which pesticides they are applying, or not understanding or even ignoring the instructions of handling and basic safety guidelines that are printed on the bottle of the pesticides [42].

It is important to point out that many studies have demonstrated that exposure to pesticides can be reduced if the farmers use PPE and fundamental sanitation practices and if they are trained in the safe handling of pesticides. Many projects have been working with farmers in IPM through educational intervention programs, improving the knowledge of pesticide safety use, recommending as a conclusion to continue with training programs especially in young people from developing countries [43, 44]. A good example was demonstrated by an educational program among 74 pesticide handlers in southern India who were evaluated in knowledge, attitude and practices (KAP) before, immediately after, and one month after training, and showed that the KAP for safe pesticide handling score greatly improved after training [45].

To improve the knowledge and assessment of occupational exposures job-exposure matrices (JEM) were developed. These JEMs were designed as an indirect way to connect occupational exposures where biological monitoring data, industrial hygiene measurements, or industry records are difficult to perform, unavailable, scarce, or inaccurate. These tools are used to estimate the quantity of pesticide used and the probability of exposure, being vulnerable to misclassification [46, 47]. For example, using a JEM in fruit farmworkers from South Africa, an association between long-term OP exposure and neurologic and neurobehavioral effects was found [48]. Moreover, a modestly increased risk of multiple myeloma was associated with occupational pesticide exposure in a large population case-control study performed in three US states [49]. In 2010, another group of researchers used the Task-Exposure Matrix (TEM) for Pesticide Use (TEMPEST) using seven decades of information (1945 – 2005) from Scotland, concluding that this JEM could be used for retrospective assessment of occupational exposure to pesticides [50]. In 2018, a generic job-exposure matrix (PESTIcides in general POPulation, PESTIPOP) for measuring occupational pesticide exposure in French general population was applied. The results showed the highest exposure probability of jobs with agriculture exposure in comparison with those jobs with non-agricultural exposure such as wood preservation, and parks maintenance, and pest control, especially agricultural jobs exposed to insecticides. The conclusion of this study suggested that this JEM can be used in future epidemiological studies [51].

1.3.3 Personal protective equipment (PPE)

Once the pesticide hazard and how it can enter the human body were understood, international guidelines were created for educating the farmers on the relationship between pesticide toxicity, exposure, and hazard. The Food and Agriculture Organization of the United Nations (FAO) created guidelines for PPE when working with pesticides in tropical climates, where among many other recommendations, the importance of education and training the farmers in handling pesticides were the main points [52]. The FAO recommends minimizing skin contamination as much as possible since this is the most likely route of exposure. For that, they recommend the use of working clothing such as coveralls, hat, gloves, eyewear, and protective footwear, as the first line of defense. The clothing must be comfortable, lightweight covering most of the body protecting against pesticide penetration [52]. The efficiency of these recommendations was successfully demonstrated in a study using water-repellent finish working coverall, which reduced the body surface exposure by a factor of approximately 95% in vineyards workers [53]. Another study showed that a larger number of Indonesian red-onion

farmers who used PPE were categorized as healthy/not sick in comparison with those who did not use it [54]. However, the effectiveness of the use of PPE for avoiding/reducing pesticide exposure is clearly reduced due to the negligence of the users themselves, this was demonstrated in a Canadian study, where farmers claimed they avoided using PPE because it was uncomfortable to wear, took too long to put on, or because farmers simply forgot about it [55]. The use of recommended PPE can be especially challenging in tropical areas due to high temperatures and humidity [52, 56].

1.4 PESTICIDE USE IN SOUTH AMERICA

The use of agrochemicals in South America has been increasing during the last decades. According to data from FAO, countries like Ecuador, Brazil, and Argentina have increased the average amount of pesticide used per area of cropland 7-fold during the last 20 years, similar numbers are reported for the rest of the South American countries [35]. Moreover, the governments are dealing with the smuggling of pesticides by which more pesticides are introduced without any control and thus becoming a potential public health problem. Moreover, despite human health hazards and environmental pollution, waste from unused and obsolete pesticides in South America is around 30 000 to 50 000 tonnes yearly according to the FAO [35]. Of concern is also the habit of storing waste close to important water bodies, such as main rivers or lakes (water for drinking), or burying waste close to communities where people probably are unaware of their existence [35]. In addition, there is a lack of training in pesticide handling and underestimation of the advantages of the use of PPE which contributes to the overuse and misuse of pesticides. Together this contributes to a large health risk for both the occupational and general population. For example, Brazil and Ecuador report an average of 12 000 and 6 400 cases of pesticide poisonings per year, respectively [57, 58]. Acute health effects related to pesticide intoxication have been reported in studies performed in Chilean and Peruvian farmers exposed to OPs [59, 60]. Besides, genotoxic damage and chronic health effects related to long-term exposure were also found in Argentinean farmers exposed to glyphosate (herbicide) among other pesticides [61] and in Colombian children exposed to atrazine (herbicide) [62]. Those studies remarked that education and the use of PPE should be promoted for reducing pesticide exposure during agricultural activities.

1.4.1 Pesticides use in Bolivia

The Plurinational State of Bolivia is located in western-central South America, with an area of 1 098 581 km^2. The main economic activity of Bolivia is the agriculture, consisting of around 2 760 000 km^2 of cultivated area, with 871 927 agricultural production units according to data from the 2013 Bolivian agricultural census [63]. Bolivia has a large climate variability which allows for the cultivation of a large number of different crops during the whole year.

Bolivia is part of the Rotterdam, Stockholm, and Basel conventions, and based on article 16 of the Political Constitution of the Bolivian State, the government must guarantee food security through healthy, adequate, and enough food for the entire population. Therefore, a National Technical Committee on Pesticides was created for the Registration and Control of Chemical Pesticides for agricultural use in 2016 [64]. This committee has the mission among other functions, to control and register the entry of pesticides into the country, avoiding the entry of obsolete, illegal, or dangerous pesticides to protect the population and the environment. However, Bolivia as a LMIC has problems with the extensive use and misuse of pesticides [65]. According to statistics from FAO, Bolivia had a 2-fold increase in the use of pesticides per area of cropland from 1.86 kg/ha in 1997 to 3.29 kg/ha in 2017 [35]. In Bolivia, pesticides are not produced, but around 500 000 tons of active ingredients per year are legally imported to the country [66]. However, pesticides can enter the internal market by smuggling, in many cases pesticides that are obsolete or banned in other countries due to their high toxicity [67]. Even though the laws exist, the control at the borders and penalties for illegal importation or sale is poor or non-existent. Therefore, pesticides are available at an accessible price to the minor consumer on the streets or in the stores, where even highly toxic pesticides can be found [67].

Studies performed in local markets where common people buy their groceries found residues of OCPs and OPs above the maximum residue limit in tomatoes from Cochabamba and Chuquisaca [68]. A recent study performed in markets from La Paz City found residues of cypermethrin, chlorpyrifos, difenoconazole, or/and lambda-cyhalothrin in lettuces, where 20% of them were above the maximum residue limit. However, the concentrations obtained did not exceed the acceptable daily intake and could be reduced by 50% after washing [69]. In both studies, the use of mixtures of pesticides was a remarkable finding where the total concentrations applied were difficult or impossible to obtain [68, 69].

One study showed that from 2007 to 2012, 70% of the committed suicides among young adult men were by the use of pesticides [70]. Other studies performed in Bolivia demonstrated that farmers use very toxic pesticides in their crops together with a lack of knowledge about safe

pesticide handling [71, 72]. Moreover, these studies reported that farmers presented symptoms of acute intoxication and neurotoxicity related to OP spraying operations [71, 72]. As a result of these studies, the same researchers together with the non-governmental organization called Plaguicidas Bolivia (PLAGBOL) [73], have been training some farmers from small Bolivian communities in the safe use of pesticides and IPM during the last decade in order to reduce these intoxications [72, 74]. Their results showed that 23 trained farmers and 47 neighboring farmers improved and maintained their training on IPM, knowledge, and pesticide handling in comparison with the control group [44]. However, from approximately 1.7 million people dedicated to agriculture in Bolivia (among livestock farming, hunting, fishing, and forestry) [66], there is a general lack of training in handling pesticides, inadequate or lack of information on hazards, and the unwillingness of farmers to accept the risks of crop loss. Moreover, the impact of this lack of knowledge on the level of exposure to pesticides is not known.

1.5 HEALTH EFFECTS LINKED TO PESTICIDE EXPOSURE

Pesticides were from the beginning developed to kill and suppress pests, but they might also be dangerous to humans and especially at high levels of exposure. Exposed individuals may develop acute pesticide poisoning (APP) a few hours after exposure, but also develop chronic diseases after a longer exposure period (years) [75, 76].

1.5.1 Acute pesticide poisoning (APP)

An APP is any health effect resulting after exposure to a pesticide or several pesticides within 48 h and could be from occupational, non-intentional exposure or suicide attempts which could end in death if the person does not receive medical attention [28, 77]. Health effects may be local (dermal and ocular) and/or systemic depending on the route/routes of exposure. These effects can be respiratory, cardiovascular, gastrointestinal, nephrotoxic, neurotoxic, or allergic reactions, depending on the amount, the time of exposure, and the type of pesticide(s) to which the person was exposed to [78, 79]. The effect on the central and peripherical nervous system is one of the most common toxic effects by many pesticides including the OPs such as chlorpyrifos and CB (insecticides). The symptoms occur rapidly, during or shortly after exposure, affecting the enzyme acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine. The inhibition of AChE causes accumulation of acetylcholine at cholinergic synapses, in both the peripheral and the central nervous system, leading to overstimulation of muscarinic and nicotinic receptors [80, 81]. This effect can be an additional

risk for people with lung disorders, convulsions, and the effects can increase with alcohol consumption. The acetylcholinesterase inhibition symptoms go from fatigue, headache, and lacrimation among others in a mild exposure, symptoms that can mimic a simple flu, making farmers or common people do not realize that they are having a health exposure-pesticide side effect. The severity of the symptoms increasing to moderate and severe poisoning by showing a marked pupils constriction, chest discomfort, inability to walk, incontinence and unconsciousness, seizures, and without the proper and opportune medical attention, the person can die [80, 82]. Studies performed in farmers from Peru and Chile exposed to mixtures of OPs and CBs showed a reduction in the cholinesterase activity in comparison with their control groups [60, 83]. Skin irritation because of dermal contact is a primary effect of pyrethroids. Intoxication by bipyridyls herbicides such as paraquat provokes severe irritation to mucous membranes of lungs and respiratory failure after 2 or 3 days. Moreover, symptoms of intoxication by chlorophenoxy herbicides such as 2,4-D and MCPA cause dizziness, mental confusion which may progress to unconsciousness [80].

1.5.2 Chronic diseases

Long-term exposure to pesticides can increase the incidence of chronic diseases, including diabetes and cancer [3, 84-86]. Moreover, the European Union (EU) together with the EFSA, has listed some pesticides, such as cyhalothrin (insecticide) and mancozeb (fungicide), as proven or possible endocrine disruptors (EDs). The list includes pesticides with evidence for ED properties in at least one study [87, 88]. As a result, in that list several pesticides have been classified as ED, among them, atrazine, bifenthrin, deltamethrin (interferes in the estrogenic activity), lambda-cyhalothrin (thyroid hormone production), and mancozeb (thyroid hormone production) have been listed [89]. EDs can affect multiple functions in many organs that respond to endocrine signals [1, 90], including decreasing fertility in both sexes, low quality of semen, demasculinization, and changes in the pattern of maturity [91]. Other pesticides have the property of affecting the production of the thyroid hormone inducing hypothyroidism (aldrin) or hyperthyroidism (mancozeb and metalaxyl) [92, 93]. Moreover, studies have associated exposure to pesticides with other chronic diseases such as rhinitis (glyphosate and chlorpyrifos), non-Hodgkin's lymphoma (NHL), asthma, chronic bronchitis (glyphosate and paraquat), and neurodegenerative disorders such as Parkinson and Alzheimer's later in life [5, 94-96].

Cancer incidence is increasing since the population is aging but also since they are more exposed to carcinogenic agents including pesticides [97]. Moreover, farmworkers are at a greater risk of developing cancer due to exposure to pesticides since they show higher cumulative exposures than the general population and especially if they are exposed to pesticides that contain arsenic or are using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which are both known as human carcinogens by the IARC (Group 1) [98]. Moreover, IARC has classified some pesticides such as malathion, glyphosate, and parathion as probable/possible carcinogenic to humans (Group 2A – Group 2B), due to multiple mechanisms including genetic damage, tumor promotion, immunotoxicity, hormonal action, and epigenetic modifications [16, 17]. In systematic reviews and meta-analyses performed by Bassil et al., Parrón et al., and Mostafalou et al., a positive association with exposure to pesticides, especially insecticides and herbicides, and a positive association of cancer incidence of the brain, prostate, breast, colorectal, pancreas, and lung was found [5, 85, 86]. In addition, an increased risk of prostate cancer was found in farmers exposed to methyl bromide (a fumigant used against a wide variety of pests) [99] and of kidney cancer in sawmills workers exposed to pentachlorophenol (herbicide) [100]. Other studies showed an increased risk of developing leukemia in farmers exposed to OPs and OCPs [86, 101].

Many studies have found an increased risk of NHL for several pesticides [86, 102]. For example, in two meta-analyses an increased risk for NHL has been linked to exposure of glyphosate-based herbicides [103] and to 2,4-D [104]. Moreover, in a cancer incidence study performed in paraquat pesticide applicators from Iowa (USA), a significantly elevated risk for NHL was found in comparison with those applicators who never applied paraquat, the study also showed that there were no associations between paraquat and other types of cancers [105]. Contrary to the above-mentioned studies, a meta-analysis did not find any associated risk between OPs (malathion, diazinon, and terbufos) with NHL [106]. Moreover, a pooled analysis of agricultural cohorts from France, USA, and Norway concluded that the associations between NHL and pesticides dependent on the subtype of NLH and on the type of pesticide. They also remarked about the necessity of more exposure-response analysis in the future for a better understanding of the association between pesticide exposure and NHL [107].

1.5.3 Chronic effects in vulnerable populations

Pesticides can induce damage in humans in periods of rapid development, such as fetal period through transplacental absorption causing in some cases teratogenic effects in the offspring,

especially in the first eight weeks after conception [108]. There are organ systems such as the central nervous system, external genitalia, and eyes that are susceptible to teratogenic effects throughout the whole pregnancy due to exposure to pesticides [91, 109]. A study performed in Egypt found that the probability of having a child with a congenital malformation was 3.4-times higher if the father was occupationally exposed to pesticides compared with the general population [110]. Additionally, birth defects were associated with exposure to glyphosate in farmer families from the Red River Valley of Minnesota, USA [111]. Other studies have shown that altered growth, such as low birth weight, prematurity, and intrauterine growth restriction, mental and motor delay, attention deficit hyperactivity disorder (ADHD), and reduced IQ, can be linked with pesticide exposure to pyrethroids and chlorpyrifos [109, 112].

1.6 MECHANISM INVOLVED IN PESTICIDE CARCINOGENESIS

Long-term exposure to pesticides has been linked to genotoxic effects in exposed humans in many studies [113-115]. For example, levels of genotoxic damage were higher in farmers from Brazil, Ecuador, and Argentina exposed to single pesticides or mixtures compared with their respective control groups [61, 116, 117]. Besides genotoxicity, the mechanisms of carcinogenesis by exposure of pesticides goes through tumor promotion, hormonal action, immunotoxicity, and epigenetic effects [118]. Here, the focus is mainly on genotoxic mechanisms (Figure 1).

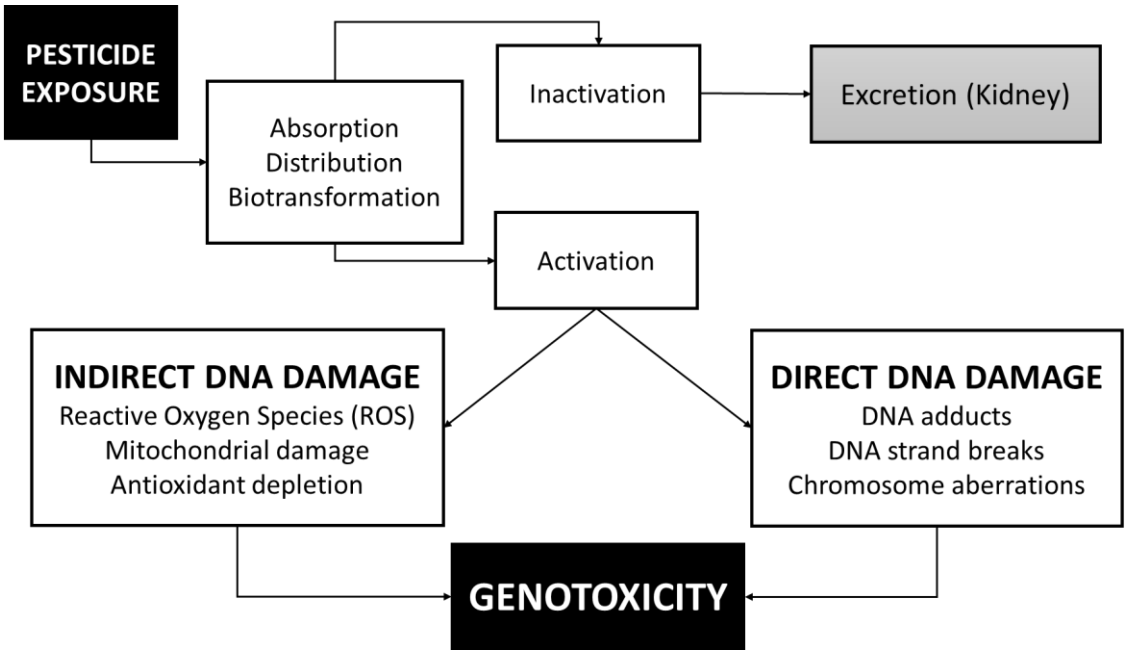

Figure 1. Genotoxicity mechanisms by pesticide exposure.

Induction of genotoxicity may lead to the formation of irreversible genomic mutations. Mutations can in turn activate oncogenes and inactivate tumor suppressor genes, leading to initiation and progression of cancer [119]. Most pesticides do not induce mutations in test systems. The pesticide cyclophosphamide has been shown to be mutagenic *in vitro*, animals, and humans due to metabolic activation into an alkylating agent. Induction of genetic changes, including sister chromatid exchanges and chromosomal aberrations, have been observed following exposure to cyclophosphamide in rats and cancer patients [120].

1.6.1 Indirect DNA damage

1.6.1.1 Reactive oxygen species (ROS) and oxidative stress

Pesticides and products of their metabolism can induce oxidative stress (disbalance between reactive oxygen species production and antioxidant mechanisms of defense), but the mechanisms by which pesticides do this is not well established [121, 122]. Oxidative stress provokes a loss of cellular integrity and function by accumulation of reactive oxygen species (ROS), this process is induced by protein oxidation, lipid peroxidation, and DNA oxidation. For example, studies in rats and hamsters have shown that some pesticides such as disulfiram and zineb (dithiocarbamates) result in oxidation of glutathione, thereby impairing the cellular response to ROS [123, 124]. Oxidative stress is considered to be a central mechanism by which pesticides induce degenerative-chronic diseases such as diabetes, atherosclerosis, autoimmune diseases, neurodegenerative diseases, and cancer [3, 125]. Studies in lung fibroblasts of hamsters have demonstrated that the cytotoxicity mechanisms of pesticides such as zineb and thiram (dithiocarbamates) include ROS formation [124, 126]. Other studies have shown a significant association between the increase of ROS and antioxidant depletion with acetylcholinesterase enzyme inhibition in farmworkers exposed to OPs and bipyridyls such as paraquat [127, 128].

Studies performed in rodents and *in vitro* (different human cells of the nervous system) have shown that OPs can induce mitochondrial damage [129, 130]. Pesticides may affect the structure of the mitochondria by damaging the internal membrane, which increases its permeability. Furthermore, energy production is halted by damage to the respiratory chain, and the Ca^{2+} homeostasis is also impaired, through the oxidation of specific thiol groups in proteins [131]. At the same time, the mitochondrial defense system that prevents lipid peroxidation is may be affected [132]. The mitochondrial dysfunction caused by OPs can promote oxidative

and genotoxic damage by triggering cell death [129]. Another study has shown the effect of low dose exposure to pesticides, such as paraquat and chlorpyrifos (among others), on mitochondria morphology in SH-SY5Y cells and its potential link with pesticide-induced Parkinsonism [133].

1.6.2 Direct DNA damage

1.6.2.1 DNA adducts

A DNA adduct is a covalent interaction between an electrophilic compound and a nucleophilic site in DNA. Due to the role of ROS as a mechanism in pesticide toxicity, oxidative DNA damage has gained a lot of attention and pesticides have been shown to induce a number of oxidative damage, including damage to individual nucleotide bases, DNA strand breaks, and the formation of adducts [121]. The most susceptible nucleic acids to react with ROS are thymine and guanine and the 8-hydroxyguanine (8-OH-G) is the most common mutagenic oxidative DNA damage. Previous studies have shown an increase in 8-OH-G in farmers exposed to mixtures of pesticides [134, 135]. Moreover, a study performed in farmers chronically exposed to OP pesticides concluded that chronic exposure can induce the stimulation of antioxidant enzymes and at the same time an increase in DNA damage [136].

1.6.2.2 Strand breaks

Pesticide exposure can provoke single-strand breaks (SSBs) and double-strand breaks (DSBs) because of direct DNA damage or indirectly by ROS formation. DSBs are the most severe since they can result in cell death (if unrepaired) or can cause chromosomal aberrations (if misrepaired) which are an early step of carcinogenesis [137]. These lesions can also block genomic replication and transcription. Many human diseases are related to these two premutagenic damages, such as ataxia-telangiectasia and neurodegeneration (SSBs), and developmental abnormalities and cancer predisposition (DSBs) [137], especially when the individuals are exposed to xenobiotics such pesticides [138, 139]. The correlation between pesticide exposure and the induction of strand breaks is described in more detail in Section 1.7.4.1.

1.6.2.3 Chromosomal aberrations

Exposure to pesticides also produces DNA lesions which may affect DNA replication and transcription. Therefore, a well-conserved DNA repair system is necessary to avoid mutations or wide-scale genome aberrations which can affect the cell viability. Chromosomal aberrations

(CAs) show abnormalities in the number and structure at the chromosomal level. A high number of CAs have been associated with an increased risk of developing cancer and chronic diseases I populations exposed to pesticides [118, 140]. Many aberrations provoke loss of chromosomal material in one of the daughter cells, a phenomenon observed as a small nucleus or micronucleus (MN) besides the nucleus. Aberrations can also disrupt cellular division itself, with a high probability of dysfunction or death [141, 142]. See Section 1.7.4.2 for more details on MN.

1.7 HUMAN BIOMARKERS

Biomarkers can be used to help detect diseases in their early stages of evolution and to evaluate the effectiveness of medical treatment. In addition, they are also used to detect people at risk of being exposed to a toxic agent (i.e. carcinogens), such as in environmental or occupational exposure. Also, they can be used to determine intrinsic individual differences, also called individual susceptibilities to these toxic agents. Once the above points have been identified, the results obtained can give a clearer idea, at the molecular level, about the etiology and pathophysiology of the disease, and can be used to find a possible solution to prevent this disease, such as the use of protective measures. [143, 144]. Even though pesticides can be measured in tissue or human fluid, the test can vary according to each chemical's properties. For that reason, biomonitoring of pesticides in biological samples is often a challenge since not all pesticides have the same half-life and both their metabolism and their excretion often vary to such an extent that many pesticides cannot be properly monitored, especially when the individuals are exposed to a mixture of pesticides [145]. In addition, for many pesticides, metabolism is not well studied.

Biomarkers are usually divided into three categories as markers of susceptibility, exposure, and effect.

1.7.1 Biomarkers of susceptibility

Biomarkers of susceptibility are factors that may make certain individuals or populations more sensitive to the influence of chemical exposure, meaning that not all individuals present the same degree of risk against a specific exposure. Several enzyme families (oxidases, reductases, etc.) participate in the biotransformation of pesticides. However, individual differences (genetic variations, polymorphisms) in the genes encoding these enzymes exist. For example,

in the enzymes that help detoxify pesticides, such as paraoxonase (PON1), cytochrome P450 (CYP) and glutathione transferases (GST). As a consequence, different metabolic capacities can be developed, reducing, their activity, and effectiveness against xenobiotics.[143, 144, 146].

1.7.1.1 Glutathione transferase (GSTs)

The human cytosolic GSTs are a superfamily of multifunctional and ubiquitous enzymes, which are classified into eight families (alpha, kappa, mu, pi, sigma, theta, zeta, and omega). Since GSTs are enzymes that act in phase II of the metabolic detoxification process, protecting the cells from attack by environmental carcinogens, ROS, and chemotherapeutic agents, genetic variants of the GSTs may impact the elimination and detoxification of pesticides [144]. A commonly studied genetic variant of GSTs is the *null* genotype of GSTM1 and GSTT1, this is a partial deletion of the gene, which leads to a total loss of enzyme activity. The *null* frequencies are usually measured by specific multiplex PCR and analyzed by electrophoresis [147, 148]. GSTM1 and GSTT1 *null* genotypes have been associated with an increased risk of developing some specific cancers, such as gallbladder and gastric cancer, in people occupationally exposed to pesticides such as OPs, CBs among others [149, 150]. This risk further increases when there is a combination of GSTM1 and GSTT1 *null* genotype and especially depending on the ethnicity and the exposure to certain genotoxic pesticides [151, 152].

Concerning GSTs and genotoxicity, an increased susceptibility to induction of DNA damage was observed in Indian farm workers exposed to different mixtures of pesticides with GSTM1 *null*, and especially with GSTT1 *null* genotype, compared with the non-*null* genotype [153]. A Bolivian study showed that farmers with GSTM1 *null* genotype had a non-significant 1.39-fold increased risk of having higher genotoxic damage compared with those with active GSTM1 [148]. Another study performed among Italian farmers showed that subjects with GSTM1 *null* genotype displayed an increase of the GSTT1 activity after exposure to pesticides, suggesting some interaction between the regulation of GSTs [154].

1.7.2 Biomarkers of exposure

Biomarkers of exposure are measurements of internal substances (parent compound itself, or its metabolites) and thus reflect internal manifestations that may result from exposure to chemicals or toxicants. Measures of the internal dose can be made in biological samples such as blood, urine, breast milk, or breath level of a chemical. The exposure biomarkers most

commonly monitored, typically reflect only those exposures occurring during the last 24 – 48 h [144, 155].

During the past years, many studies have been performed to investigate the levels of exposure to pesticides in different populations. However, each pesticide has a different half-life, some of them are quickly excreted during the first 24 h (short half-life e.g. OP), making biomarker measurements a challenge. On the other hand, some pesticides have the property of bioaccumulation in the fatty tissues and are persistent in the body during long periods of time. This is the case of OCPs with a slow elimination rate that can be measured in human breast milk and adipose tissue among others. For example, a systematic review performed in 2015, revealed that the amounts of OCP in human breast milk in Asian, African, and South American countries were higher than those of European countries [32]. However, the elimination rate of pesticides in blood for many other pesticides, such as OPs, CBs, and pyrethroids, is quite fast and may represent a lower concentration than what was truly absorbed by the body [144, 145]. For these reasons, probably, the most common measurement for pesticide metabolites is analyzed in urine samples.

1.7.2.1 Urine pesticide metabolites (UPMs)

Metabolites of certain pesticides excreted in urine have been used as a biomonitoring measure of exposure in different countries [156-158]. This is a simple, non-invasive, and quick to analyze technique, which only requires a sample of the first urine in the morning or a 24 h urine collection. UPMs as biomarkers of exposure should be selective for each pesticide or group of pesticides measured. For example, OPs are hydrolyzed in six dialkylphosphates (DAPs), pyrethroids metabolites that are excreted in the urine are 3-PBA, DCCA, F-PBA, and DBCA, among other pesticide metabolites [159]. However, UPMs have to be stable, without any artifactual formation of the measured compound [160]. Many studies have been performed for detecting exposure to pesticides using UPM. For example, a study showed an evident reduction of lung function in a Canadian population in correlation with high concentrations of urine pyrethroids metabolites[161]. Another study found 2.5 times higher UPM levels of OP pesticides in pregnant women living in an agricultural area compared to the US general population [98]. Moreover, another study using UPM performed in adults from Shandong, China showed a wide exposure to chlorpyrifos in farmers compared with non-exposed urban adults [162].

1.7.3 Biomarkers of effect

Biomarkers of effect identify changes in biological function caused in response to exposure to chemicals or agents. These biomarkers are more directly related to the health effects that can potentially cause, such as chronic diseases. [144]. Unlike biomarkers of exposure that are mostly specific for exposure chemicals, biomarkers of effect are often nonspecific for the substance in question. This feature may suggest that they have a strong ability to reflect complex exposures, such as mixtures of pesticides, and therefore, they should also be able to include sequential and summative exposures over time. On the other hand, its use in complex exposures could also be used to identify active components in mixtures as a consequence of combined exposures and also to identify the adverse effects that this mixture can cause [163]. For example, changes in the hemoglobin synthesis and other hematological effects have been found in populations exposed to OCPs [164]. In other studies, the inhibition of AChE has been observed in farmers and their children exposed to OP pesticide [165, 166]. Moreover, products of oxidative DNA damage can be used for biomonitoring. For example, a positive correlation between oxidative stress biomarkers (8-OH-G levels, malondialdehyde, and isoprostane) and oral exposure to mixtures of OP pesticides was found in male farmers [167].

1.7.4 Biomarkers of genotoxic effects

These biomarkers are usually considered the first biological changes as a consequence of a carcinogenic process. They can be measured for human biomonitoring usually in lymphocytes of peripheral blood using two gold standard techniques: the comet assay and the cytokinesis-block micronucleus cytome assay [168]. There are also other methods for detecting DNA damage. Quantification of DNA adducts can be done at target organs for monitoring external exposure and can integrate measurements of the effects of factors like absorption, distribution, metabolic activation, and/or DNA repair [169]. Another example is the measurement of γH2AX which also was used in the present thesis *in vitro*. The two gold-standard assays and γH2AX measurements are described in more detail below.

1.7.4.1 Comet assay

The comet assay is a fast and sensitive technique, used to detect DNA strand breaks in individual cells, in response to DNA damaging agents [170]. The single-cell gel electrophoresis (SCGE) or the alkaline comet assay is well-known for measuring genomic stability changes, being one of the most accepted techniques by many governmental regulatory agencies. Since 2014, the OECD guidelines included the comet assay as a part of the assays for testing

chemicals (Test No. 489) [171]. The test detects SSBs and DSBs, alkali labile sites, and DNA cross-linking in individual cells [139, 172]. In a study performed in Brazilian farmers exposed to mixtures of pesticides a statistically significant increase in strand breaks was found in comparison with the control group [113]. In two studies performed by How et al. in 2015, and Carbajal et al. in 2016, were shown that chronic exposure to OPs and pesticide mixtures among farmers increased the level of strand breaks 2-fold in comparison with the non-exposed groups [173, 174].

1.7.4.2 The cytokinesis-block micronucleus cytome (CBMNcyt) assay

This is a powerful tool for the measurement of chromosomal aberrations. Micronuclei (MN) refers to a third nucleus or more formed during the metaphase/anaphase transition of mitosis after chemical exposure, as a result, one of the daughter cells doesn't have a part or all chromosome [143]. Nuclear abnormalities such as MN, nuclear outbreaks (NBUD), and nucleoplasmic bridges (NPB), are manifestations of chromosomal instability that are commonly observed in cancer, which is why they are considered biomarkers with a genotoxic effect. These abnormalities provide measurements of poorly repaired DNA breaks, defective sister chromatid separations, absence or dysfunction of telomeres, formation of repair complexes, and DNA amplification. [175]. As examples, high frequencies of MN and other nuclear abnormalities were detected in Brazilian, Mexican, and Bolivian farmworkers exposed to mixtures of pesticides [148, 174, 176]. Moreover, studies and reviews performed by Bolognesi et al in 2016 and Bonassi et al in 2011, showed that a high frequency of MN in peripheral blood lymphocytes was associated with an increased risk to develop cancer in populations occupationally exposed to pesticides compared with their respective control [177, 178]. Additionally, a recent study showed that soybean farmers who were working with long-term exposure to low levels of complexes mixture of pesticides showed significantly increased levels of MN compared with the control group [115].

1.7.4.3 γH2AX

Histone 2AX (H2AX) is a key protein in the activation of DNA damage response and DNA repair. H2AX is activated by phosphorylation, named γH2AX, by several kinases in response to DNA damage such as DNA adduct and strand breaks [179]. The induction of γH2AX can be visualized as foci by immunocytochemistry and is one of the earliest events detected following exposure to DNA damaging agents [180]. γH2AX has been used as a real-time method to image DNA damage, for biomonitoring DNA damage in cancer treatment *in vivo*. In a study performed by Cornelissen et al., anti-γH2AX probes were used as a non-invasive imaging method to monitor DNA damage using a mouse xenograft model of human breast

cancer treatment [181]. Besides, Sak et al., have shown high sensitivity of γH2AX in *in vitro* radiations, summarizing the possibilities of using γH2AX as a clinical biomarker during radiotherapies such as the monitoring of drug effects on DNA damage responses pathways after *in vivo* drug exposure and a subsequent *in vitro* radiation [182]. Moreover, since the risk of exposure to complex mixtures is difficult to estimate. In order to improve health risk assessment, researchers have been measured in HepG2 cells, the additive effects of binary exposure to complex mixtures of polycyclic aromatic hydrocarbons (PAHs) with other mixtures in urban air PM extracts (e.g. dibenzo[*a,l*]pyrene and benzo[*a*]pyrene). Their results showed the effective use of γH2AX in the DNA damage signaling and DNA damage response, and concluded that γH2AX can be used as a biological marker for analyses of complex mixtures of PAHs [183, 184]. Therefore, the γ-H2AX assay could represent an effective approach for quantifying DNA damage by pesticide exposure.

2 AIM OF THE STUDY

The overall aim of this project was to evaluate the correlation between exposure to pesticide mixtures and genotoxicity for the population in Bolivia. The specific aims of the included studies were as follows:

- To characterize the lifestyle factors, handling, and exposure to pesticides in a Bolivian agricultural population (Paper I).

- To investigate the correlation between exposure to pesticides, genetic susceptibility, and genotoxic effects in a Bolivian agricultural population (Paper II).

- To determine possible mixture effects for genotoxic damage of commonly used pesticides in our studied Bolivian population (Paper III).

3 METHODOLOGIES

This thesis combines epidemiological studies to show the effects of the mixtures of pesticides in the studied populations (papers I and II), and in *in vitro* studies in order to improve the understanding of possible effects of those mixtures using human liver HepG2 cells (paper III). This chapter provides an overview of the different methodologies used in the three papers.

3.1 PAPER I AND II

3.1.1 Design and study areas

Paper I and II had a cross-sectional study design based on populations of three agricultural communities of Bolivia. Taking into account that cross-sectional studies can be done at one determined point of time and the fact that the obtained data can be used to create new theories or hypotheses, this design fits perfectly in this thesis, making the first approach to study the pesticide exposure and their effects on this population.

The study was carried out in three agricultural Bolivian communities where, according to the Bolivian census, 65 - 79% of the population is farmers [63, 185]. Sapahaqui (Com1) located at 3134 m above sea level in the second municipal section of Loayza province in the Department of La Paz, Villa Bolivar and Villa 14 de Septiembre (Com2 and Com3, respectively) at 200 m above sea level in the third municipal section of the Chapare Province in the Department of Cochabamba (Figure 2). Their agricultural production is based mainly on vegetables, citrus, and other kinds of fruits [63].

Figure 2. Map of Bolivia and the three farming communities studied. Sapahaqui (Com1) located in La Paz, Villa Bolívar (Com2) and Villa 14 de Septiembre (Com3), both located in Cochabamba.

3.1.2 Participant recruitment and ethical considerations

The study population was recruited for participating in the project with the help of health promoters and local government authorities who were previously contacted by our team. They organized meetings where the people were informed orally about the project and after that, people (farmers and no farmers) who were interested in participating booked a date for being included in the study. Once the dates were fixed, our team traveled to the communities to perform the fieldwork, and we informed them again about the project and our aims. Each participant voluntarily signed informed consent and a copy of the information sheet was given to them. They were informed about their right to leave the study at any time they wanted. People who were included in the study were women and men with an age range of 17-70 years old who had lived in the area at least five years ago. These ages represent years that farmers, in general, are active in Bolivia. In total, 297 people participated in the study.

Ethical permits were obtained from the national ethics committees of Bolivia and Sweden, and the study was conducted following the principles of the Helsinki Declaration. An individual code was assigned to each participant and the original data was saved in a place where only the principal investigators have access to the identifiers.

3.1.3 Evaluation of pesticide exposure

In order to get an overview of the pesticide exposure situation in the three communities, we combined two sources of information. The first was by collecting information using an exposure survey, where the most frequently used pesticides, among other questions, were assessed. The second source was by collecting urine samples for measuring pesticide metabolites. Both methodologies are explained in the next pages.

3.1.3.1 Exposure survey

To characterize the exposure situation concerning lifestyle factors and behaviors related to the use and handling of pesticides, face-to-face interviews were done by trained members of the staff from the Genetic Institute at Mayor of San Andres University (UMSA) in La Paz City, Bolivia. A survey based on a questionnaire employed previously in Bolivian farmers [65, 186] with some modifications for the current study was applied. The survey contained closed- and open-ended questions related to their general personal information and lifestyle, use of pesticides, and PPE and with a special section aimed at women's health. Besides, questions related to acute health effects and chronic diseases by pesticides were included.

3.1.3.2 Collection of urine samples and urine pesticide metabolite analysis

We collected spot samples of the first urine in the morning in empty sterile polypropylene containers. These were given to the participants the day before the sample collection with some hygienic recommendations for avoiding contamination. The urine samples were collected before the start of the interview, aliquoted, stored at -18 °C, transported to the Genetic Institute, UMSA, La Paz City, Bolivia, and then shipped to Lund University in Sweden to the Division of Occupational and Environmental Medicine, where concentrations of ten UPMs were analyzed. The samples were adjusted for the degree of dilution, in this case by urine creatinine. The measured UPMs (Table 1) were 3-phenoxybenzoic acid (3-PBA), the sum of cis/trans 3-(2,2-dichlorovinyl)−2,2-dimethylcyclopropane carboxylic acid (DCCA), chloro-3,3,3-trifluoro-1-propen-1-yl]−2,2-dimethylcyclopropanecarboxylic acid (CFCA) and 4-fluoro-3-phenoxybenzoic acid (4F3PBA) for measuring pyrethroids, 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methyl phenoxy acetic acid (MCPA) for measuring phenoxy herbicides, hydroxy-tebuconazole (TEB-OH), 5-hydroxytiabendazole (5-OH-TBZ) and 3-hydroxy-pyrimethanil (OH-PYR) for measuring fungicides, and 3,5,6-Trichloro-2-pyridinol (TCP) for measuring the organophosphate chlorpyrifos. Briefly, using a β-glucuronidase/arylsulphatase, the urine samples were de-conjugated and prepared with solid-phase extraction. Following a modified method and using a liquid chromatography-triple

quadrupole linear ion trap mass spectrometer, a quantitative analysis was performed [160, 187]. Limits of detection (LOD) were 0.10 ng/mL for all the metabolites except for 5-OH-TBZ that was 0.05 ng/mL. For metabolites in which concentrations were under LOD, LOD/2 was used for statistical analysis.

Table 1. Urine pesticide metabolites analyzed in the thesis

UPM	Pesticide(s)	Chemical family	Type of pesticide
2,4-D, MCPA	Phenoxy herbicides	Phenoxy acetic acid	Herbicide
TCP	Chlorpyrifos	Organophosphate	Insecticide
3PBA, DCCA	Cypermethrin, Permethrin, and Cyfluthrin	Pyrethroid	Insecticide
4F3PBA, CFCA	Bifenthrin and Cyfluthrin	Pyrethroid	Insecticide
5-OH-TBZ	Thiabendazole	Benzimidazole	Fungicide
OH-PYR	Pyrimethanil	Aminopyrimidine	Fungicide
TEB-OH	Tebuconazole	Triazole	Fungicide

3.1.4 Evaluation of metal exposure

Most people from rural areas in Bolivia do not have access to clean drinking water, many of them have to drink water from different natural sources such as the nearest river, underground water, and/or spring water [185]. Since previous studies showed that some parts of Bolivia have high levels of metal in surface and groundwater [188, 189], 20 urine samples from farmers that claimed to consume groundwater from a well or water from the river were selected for metal analysis. Samples were analyzed at the Institute of Environmental Medicine, Karolinska Institutet, Sweden by using Agilent 7700x Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) [189, 190] (Agilent Technologies, Tokyo, Japan), which is capable of detecting metals at very low concentrations, equipped with an ORS collision/reaction cell to minimize spectral interferences. Concentrations of cadmium (Cd), lead (Pb), and arsenic (As) were determined and normalized using the urinary density.

3.1.5 Evaluation of genotoxic damage and glutathione transferase polymorphism

3.1.5.1 Collection of blood samples

On the day of the interview two whole blood samples were taken, one in heparin and the other in EDTA vacutainer tubes with approximately 3 mL for each tube. After the respective codification, samples were stored at 4 °C and transported to the Genetic Institute, UMSA, La Paz City, Bolivia. Within 20 h of collection, the samples were subjected to different techniques for genotoxicity and genotyping assessment.

3.1.5.2 Alkaline comet assay in peripheral blood lymphocytes

The comet assay can be applied to any cell type, detecting biomarkers of interest such as single- and double-strand breaks or DNA repairing capacity in its alkaline version [170, 191]. Briefly, isolated single cells (lymphocytes) were embedded in agarose and then lysed with lysis solution and non-ionic detergent for removing their cell membranes and all cell contents except the DNA attached to a nuclear scaffold. Next, the nucleus was treated with a high alkaline solution (pH $\geq$ 13) for unwinding the supercoiled DNA. Subsequently, electrophoresis was run, and undamaged DNA sequences remain closer to the nuclear scaffold, and DNA breaks migrate towards the anode, resembling the shape of a comet. To minimize artifactual DNA damage, the whole procedure was performed in dimmed light and ethidium bromide was used for staining the samples. Thereafter, using specific software, 100 comets were scored from each sample and evaluated based on the length of DNA migrated in tail, expressed as a percentage of DNA in tail, tail length, and tail moment. The most recommended parameter generally is the percentage of DNA in tail for being easy to interpret [192].

3.1.5.3 Cytokinesis-block micronucleus (CBMN) assay

The application of this assay in lymphocytes is a well-known technique for measuring chromosomal damage. Using morphological criteria, the CBMN assay makes possible the detection of genotoxic and cytotoxic effects, such as MN that originated from chromosome breaks or whole chromosome loss that lag during nuclear division. Other chromosomal aberrations that can be measured are NPBs that show DNA misrepair or telomere end-fusions, NBUDs that show elimination of amplified DNA and/or DNA repair complexes. This technique also allows for measuring cytostatic effects and cytotoxicity (cell division inhibition, and necrosis and apoptosis respectively) [175, 193, 194]. MN must be scored after nuclear division in binucleated cells that are in the telophase stage and using cytochalasin-β for

blocking the cytokinesis allows for this. Many studies have shown that MN formation is a good predictor of cancer risk because it is associated with early events in carcinogenesis [178, 195]. Here, whole blood samples were cultivated for 72 h in medium with phytohemagglutinin M, antifungal, and antibacterial solutions at 37 °C. Cytochalasin-β was added after 44 h. Lymphocytes were collected and a hypotonic solution was added for swelling the cytoplasm, after that, samples were washed in fixative methanol and loaded on microscope slides. Subsequently, slides were stained with Giemsa for microscopic evaluation. The scoring was done on 1500 binucleated lymphocytes per sample following scoring recommendations [193, 196].

3.1.5.4 Genetic variants of glutathione transferases

In paper II, following a protocol described by Abdel-Rahman et al. with modifications by Tirado et al. [147, 148], the frequency of GSTM1 and GSTT1 polymorphisms were analyzed using a multiplex PCR approach. The following PCR primers were used: GSTM1, 5′-GAACTCCCTGAAAAGCTAAAGC, and 5′-GTTGGGCTCAAATATACGGTGG; GSTT1, 5′-TTCCTTACTGGTCCTCACATCTC, and 5′-TCACCGGATCATGGCCAGCA. As an internal control, exon 7 of the CYP1A1 gene (312 bp) was co-amplified using primers 5′-GAACTGCCACTTCAGCTGTCT and 5′-CAGCTGCATTTGGAAGTGCTC. Electrophoresis was then performed with ethidium bromide stain, and the scoring was evaluated by the presence or absence of bands at 480 (*GSTT1*) and 215 (*GSTM1*) bp, respectively.

3.1.6 Use of text mining for analyzing mode of action

Text mining is a useful tool that helps researchers to reduce the searching literature time-consuming process by classifying abstracts, of the desired chemical(s), by their similar toxicological profiles for being assessed in groups rather than as a single compound. To analyze the literature related to the measured pesticides, the text mining tool CRAB3 (http://crab3.lionproject.net) was employed. This is an automated text-mining tool used to identify information on the carcinogenic mode of action (MOA) for any chemical of interest. The carcinogenic taxonomy covers non-genotoxic and genotoxic MOAs and can thus be used as a support in risk assessment and for grouping chemicals [197, 198]. Since our study population used and were exposed to a large number of pesticides that were classified as possible carcinogenic to humans by IARC [199] and US EPA [200], we used CRAB3 to get a

better understanding of the carcinogenic MOA of the measured pesticides. With that in mind, the 10 most frequently used pesticides were analyzed and from the 14,214 abstracts found in PubMed, 30% were relevant for the classification of genotoxic and non-genotoxic MOAs. Based on these abstracts, and in agreement with reports published by IARC and US EPA, the most common carcinogenic MOAs associated with pesticides were mutations, cell proliferation, and oxidative stress. Applying an assessment score found a slightly higher proportion of genotoxic and non-genotoxic MOA for pesticides found in Com3, especially for cell proliferation and oxidative stress, compared with the other two communities.

3.1.7 Statistical methods

For the population study, an Excel database was created where all the information was collected and codified. All the participants were assigned with an individual number (code) to avoiding the use of names. Once all the questions and answers were codified (variables dichotomic or continuous), the database was transferred to Statistical Package for the Social Sciences software (SPSS Statistics 25) for statistical analysis. The choice of statistical tests was based on the central limit theorem, which allows the use of parametric test when the sample size is large enough to consider that the sample means are approximately normally distributed (usually $n \geq 30$). Our sample sizes ranged from 30 to 297. Accordingly, we used Student's t-test or one-way ANOVA with Dunnett's or Bonferroni's test for multiple comparisons. Significances were determined with a $p \leq 0.05$.

To test if there was an association between the use of PPE and the handling of pesticides and the risk of exposure to pesticides, a score called “protection and handling index” (PHI) was created. The PHI score was based on international recommendations for the use of PPE and handling [52, 201], and the use of a PPE or following recommendations was assigned a point. For example, if the person was using an overall while spraying, they were given 3 points. The maximum point was 16 and represented the use of recommended PPE and best behavior (Table 2). For the statistical analysis, if the score obtained by an individual was above the median, they were classified as well-protected.

Table 2. Values for calculating the PHI score.

Variable	Farmer answer	Points
Overall	Yes	3
	No	0
Hat	Yes	1
	No	0
Mask/Scarf	Yes	1
	No	0
Boots	Yes	1
	No	0
Gloves	Yes	1
	No	0
Glasses	Yes	1
	No	0
Apron	Yes	1
	No	0
Pesticide information source	Read labeled information/Agricultural engineer	1
	Own experience/Pesticide seller	0
Amount of pesticide used in each application	Recommended amount	1
	Doesn't measure at all	0
Chew coca meanwhile spraying	Yes	0
	No	1
Change spraying clothes	Yes	1
	No	0
Storage spraying clothes	Outside the house separately	1
	With all other clothes inside the home	0
Wash clothes for spraying	Separately	1
	With all other clothes	0
Storage pesticides and equipment	Outside the house	1
	Inside the house	0

To classify the level of exposure, individuals who had UPM concentrations above the 75th percentile were classified as highly exposed. The testing was performed by logistic regression and including confounding factors (gender, age, BMI, source of drinking water, and geographical area) to reduce the bias in our study. Confounders were selected based on expert's knowledge.

To analyze if exposure to pesticides was associated with an increased risk of genotoxic damage, linear (Pearson) and logistic regression were applied. As above, the exposure levels were classified as high if UPM concentrations were above the 75th percentile. Similarly, individuals

with levels of genotoxicity above the 75th percentile were classified as having increased levels of genotoxic damage.

To test if we could identify typical profiles of pesticide exposure and if they were associated with an increased risk of genotoxic damage, the UPM data were clustered using Ward's hierarchical linkage. All 10 UPMs were included and exposure was classified as binary being either below or above 75^{th} percentile. As a result, 8 clusters were identified, wherein cluster 0 was included all the participants with lower exposure levels to any pesticide ($< 75^{th}$ percentile), and for that reason, it was used as the reference category. After that, logistic regression and one-way ANOVA with Dunnett's testing were used to analyze the resulting clusters.

3.2 PAPER III

3.2.1 Pesticide selection criteria and mixture compositions

The exposure assessments in papers I and II showed that Bolivian farmers were highly exposed to some pesticides. Based on those results, and to study possible mixture effects of the most common pesticides, eight of the most frequently used and those found at highest urinary concentrations were chosen to be tested alone or as mixtures *in vitro* (Table 3). Except for paraquat and glyphosate that were diluted in deionized sterile water, dimethyl sulfoxide (DMSO, 99%, from Sigma Aldrich) was used for diluting all pesticides. Single pesticides were tested at up to 0.1 mM except for glyphosate, which was tested up to 0.07 mM due to lower solubility. Four mixtures were made, three of them were prepared to represent typical community exposure profiles (mixtures U1, U2, and U3) and one mixture was based on the overall most frequently used pesticides (mixture S1) (Figure 3). For all the mixtures, cypermethrin was selected as a representative of all the pyrethroids (e.g. bifenthrin, cyfluthrin, cypermethrin, and permethrin). The sum of concentrations was used in all experiments. The same approach was recently used for assessing the genotoxicity of pesticide mixtures identified in the diet of the French population in HepG2 cells [202, 203]. All mixtures were used at up to 1 mM except mixture S1 whose maximum concentration was 0.2 mM due to solubility issues with paraquat.

Table 3. The pesticides tested *in vitro.*

Source of information	Pesticides	CAS number[a]
UPM	2,4-D	94-75-7
	Chlorpyrifos[b]	2921-88-2
	Cypermethrin[b]	52315-07-8
	Tebuconazole	107534-96-3
Survey	Glyphosate	1071-83-6
	Methamidophos	10265-92-6
	Paraquat	75365-73-0
	Profenofos	41198-08-7

[a]All the pesticides were PESTANAL™ analytical standard, with purity ≥ 90% and purchased from Sigma-Aldrich.
[b]Pesticides found in both sources of information (UPM and survey).

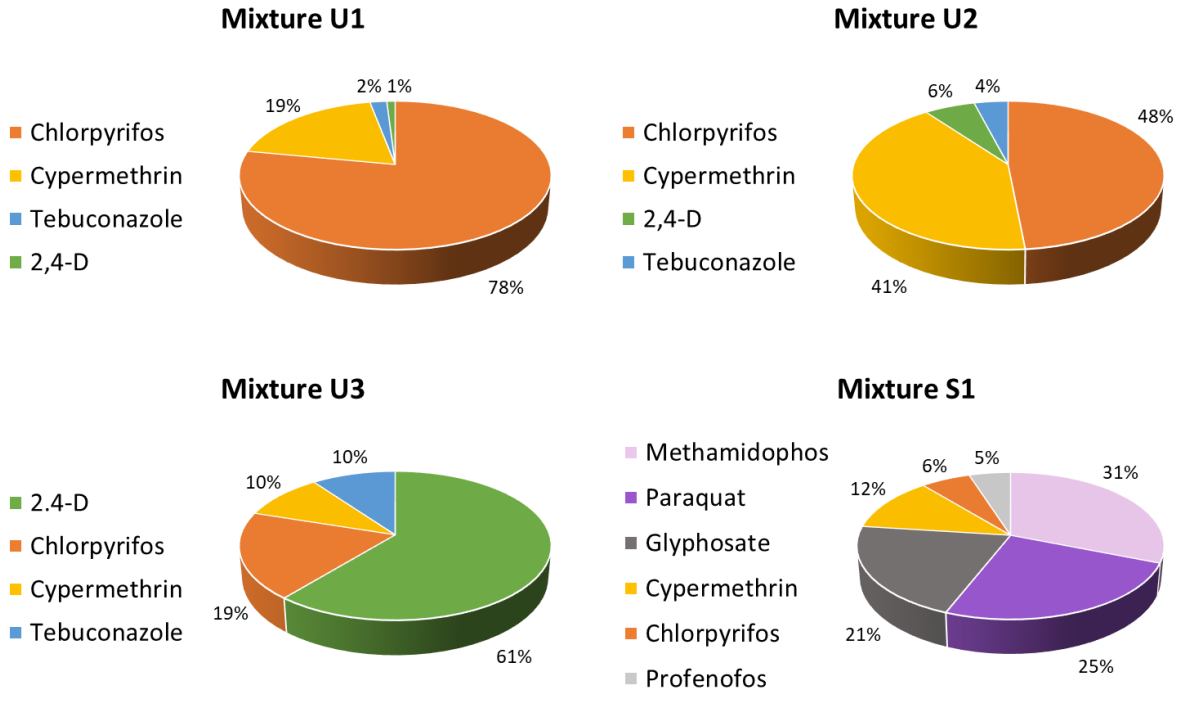

Figure 3. Overview of the prepared pesticide mixtures. The pie charts show the relative proportion of each pesticide.

3.2.2 Cell model

3.2.2.1 HepG2 cells

Pesticides and all kinds of xenobiotics can enter the body through many routes. The oral route is one of the most important for the general population, and exposure through dermal absorption and inhalation probably the most important for occupational settings. As motivated below, we choose to use an *in vitro* model based on liver cells rather than skin cells or lymphocytes.

After oral exposure, these substances are absorbed by the intestines, transported via the bloodstream to the liver, where the hepatocytes carry out biotransformation reactions that are essential for the detoxification process [204-206]. Biotransformation has also been shown to be important for some of the toxic effects associated with exposure to pesticides, such as the production of reactive oxygen species (ROS) [121, 122]. One of the most common liver cell lines to study toxicity *in vitro* is the human hepatocellular carcinoma HepG2 cells. These cells were chosen based on their metabolic competence and because the genotoxicity of many classes of environmental carcinogens, including pesticides, have been extensively studied in this cell line [207-209]. Also, and because genotoxicity was one of our main interests, activation of DNA damage signaling through phosphorylation of Chk1 at Ser-317 (pChk1) and H2AX at Ser-139 (γH2AX) in HepG2 cells has been shown to be good markers for genotoxic and carcinogenic potency of environmental pollutants including polycyclic aromatic hydrocarbons [184, 210].

HepG2 are epithelial cells derived from the liver tissue of a young 15-year-old Caucasian male [211]. These cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell culture was performed in Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM) all from Gibco by Life Technologies, Stockholm, Sweden. Cells were kept at 5% carbon dioxide / 95% air in an incubator at 37 °C.

3.2.3 Evaluation of cell viability

3.2.3.1 Alamar blue assay

The Alamar blue assay is one of the most referenced cytotoxicity assays. The assay is based on the capacity of healthy cells to maintaining their reducing environment converting resazurin

which is a blue non-fluorescent dye to resorufin that is a highly fluorescent pink dye [212]. Cells with conserved metabolic activity convert resazurin to resorufin, therefore the fluorescence and the color change of the media can be detected by measuring the increasing fluorescent signals at excitation wavelength 530-560 nm and emission wavelength 590 nm. This is a highly sensitive assay that provides time-course measurements, is permeable through cell membranes, and can be used with different cell models. Alamar blue is considered safe for the user and the environment since it is not toxic or radioactive. One of the disadvantages of this technique is a possible fluorescence interference that can be detected from the currently tested compounds. This technique can also be used as a cellular proliferation assay since the health status of the cells can be assessed based on the cell number and their metabolic activity [213].

3.2.4 Evaluation of reactive oxidative species generation

3.2.4.1 DCFH-DA assay

The evaluation of intracellular reactive oxidative species (ROS) was measured through the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay. This is a low-cost assay, easy to use, highly sensitive to redox state changes of the cell and the changes in ROS can be followed over time [214]. DCFH-DA is de-acetylated to $DCFH_2$ and converted to DCFH anion by intracellular hydrolyzation, this compound subsequently can be oxidized by intracellular ROS to DCF. DCF is a fluorochrome that can be detected with fluorometric techniques after excitation with blue light (around 488 nm) which emits green light (around 525 nm) that can be measured by a plate reader. The technique can react with alkoxyl, hydroxyl, peroxyl, and carbonate radicals and with hydrogen peroxide, but cannot distinguish which type of ROS is detected [215, 216].

3.2.5 Evaluation of gene expression

3.2.5.1 Real-time PCR

One variant of the polymerase chain reaction (PCR) is the real-time PCR (RT-PCR) which can be used for measuring expression levels of genes of interest. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). This kit avoids the use of hazardous reagents such as phenol or chloroform and minimizes DNA and protein

contamination when purifying RNA. Using a Nanodrop platform, total RNA was quantified, and quality checked. Subsequently, total RNA was used to generate cDNA using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following protocol. Using SYBR® green qPCR Master Mix with detection on a QuantStudio 5 Real-Time PCR System (both from Applied Biosystems) gene expression was analyzed using specific primers for oxidative stress (*SOD1, CAT1, GPX1,* and *HMOX1*) and DNA damage response (*CDKN1A*) genes and with *GAPDH* as a housekeeping gene. SYBR Green is a fluorescent dye with the property of binding to all newly synthesized double-strand DNA at each round of amplification. The fluorescence will accumulate and measured at the end of every PCR cycle. The amount of fluorescence is proportional to the quantity of double-strand DNA in the reaction (given as a C_T value). Relative gene expression quantification was performed based on the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) [183, 217]. Also, a melting curve analysis can be used for confirming the specificity of the RT-PCR reaction, discriminating between primer-dimers and false amplification due to contamination.

3.2.6 Evaluation of genotoxicity

3.2.6.1 Mini-gel comet assay

The mini-gel comet assay used the same method described above for the human samples, but 3 samples (gels) were loaded onto one single microscope slide, giving the same reliability as the classic alkaline comet assay but saving time. In short, after exposure, trypsin was used for harvesting the cells, hydrogen peroxide was used as a positive control (25 µM) and the mini-gel comet assay was performed following the protocol described by Di Bucchianico et al [218, 219]. Samples were fixed with methanol and stained with SYBR® green. The scoring was performed as described above for paper II.

3.2.6.2 Western Blot

Protein immunoblot or Western Blot, is an important technique for the immunodetection of proteins, especially proteins that are at low abundance [220]. This immunoassay uses specific antibodies to identify proteins in an electrophoresis gel that has been separated by their size. Then the gel is placed next to an absorbent membrane of nitrocellulose or PVDF (polyvinylidene fluoride) where the proteins are transferred by an electrical current by migration, which is known as western blotting or protein blotting. Furthermore, the membrane is processed with specific antibodies of interest, and using secondary antibodies and detection

reagents the proteins can be visualized [220]. Here, primary antibodies from rabbit were used against Chk1 phosphorylated at Ser-317 (pChk1, 1:300 in 5% BSA), H2AX phosphorylated at Ser-139 (γH2AX, 1:500 in 5% BSA), and the endogenous control Cdk2 (1:4000 in 5% of milk). Secondary rabbit antibodies were prepared following manufacturer recommendations for pChk1 (1:1000), γH2AX (1:1000), and CDK2 (1:10000) all diluted in 5% milk. Immediately an X-ray film cassette was prepared and exposed films/membranes to enhanced chemiluminescence (ECL). Films were developed in a dark room, after that the densitometric band analysis was performed to convert qualitative band intensities into quantitative information [183, 221].

3.2.7 Statistical methods

GraphPad Prism 8 (GraphPad Software LLC) was used for all the statistical analyses. At least three independent experiments were done, for all the techniques, and mean values and standard errors were determined. Non-linear regression was used to determine EC_{50} values for the Alamar Blue assay. One-way ANOVA was used for finding significances and Dunnett's or Kruskal-Wallis' tests were applied for multiple comparisons between exposures and the reference control.

4 RESULTS AND GENERAL DISCUSSION

4.1 POPULATION STUDIES IN PAPER I AND II

4.1.1 Population characteristics

A total of 297 people participated in the study, 130 women (44%) and 167 men (56%). Many of the participants were farmers (94%, n = 275) and had been working in the field for eight years or more. We could see that women to a higher degree than men were doing other activities not related to farming (non-farmers). In general, we did not find big differences in the population's characteristics among the communities. In agreement with previous studies performed in agricultural communities in other LMICs [222, 223], the education level in this population was low, 62% had only gone to primary school, and 12% never went to school. This was especially observed among women. According to the international guidelines concerning BMI measurements [224], a larger number of women were obese (BMI ≥ 30, 33%) compared to the men. None of the participants were heavy smokers or drinkers, but cigarettes and alcohol consumption were more frequent among men. Since the municipal water access is limited in some parts of Bolivia [225] and there is evidence that the water of some natural resources contains traces of metals [189], only 39% of our studies population could buy clean water in bottles and/or have access to municipal water. As a result, the majority used water from other sources like the nearest river, spring, or underground water for drinking and cooking.

4.1.2 Usage and handling of pesticides and PPE

We found that farmers in all 3 communities used a large number of different pesticides. 75% of the farmers mixed at least two pesticides for spraying the same crop. Methamidophos, paraquat, and glyphosate were the most commonly applied pesticides (Figure 4). Com2 and Com3 used paraquat and glyphosate more often, while Com1 applied chlorpyrifos and profenofos to a larger extent. Since Com2 and Com3 are located in the tropical area of Bolivia, farmers sprayed more than 20 days per month. In comparison, farmers in Com1, which is situated in a temperate region, only sprayed 2 to 10 days per month. Men sprayed more days per month and more hours per day than women. Notably, 26% claimed not to measure the amount of pesticide used for spraying.

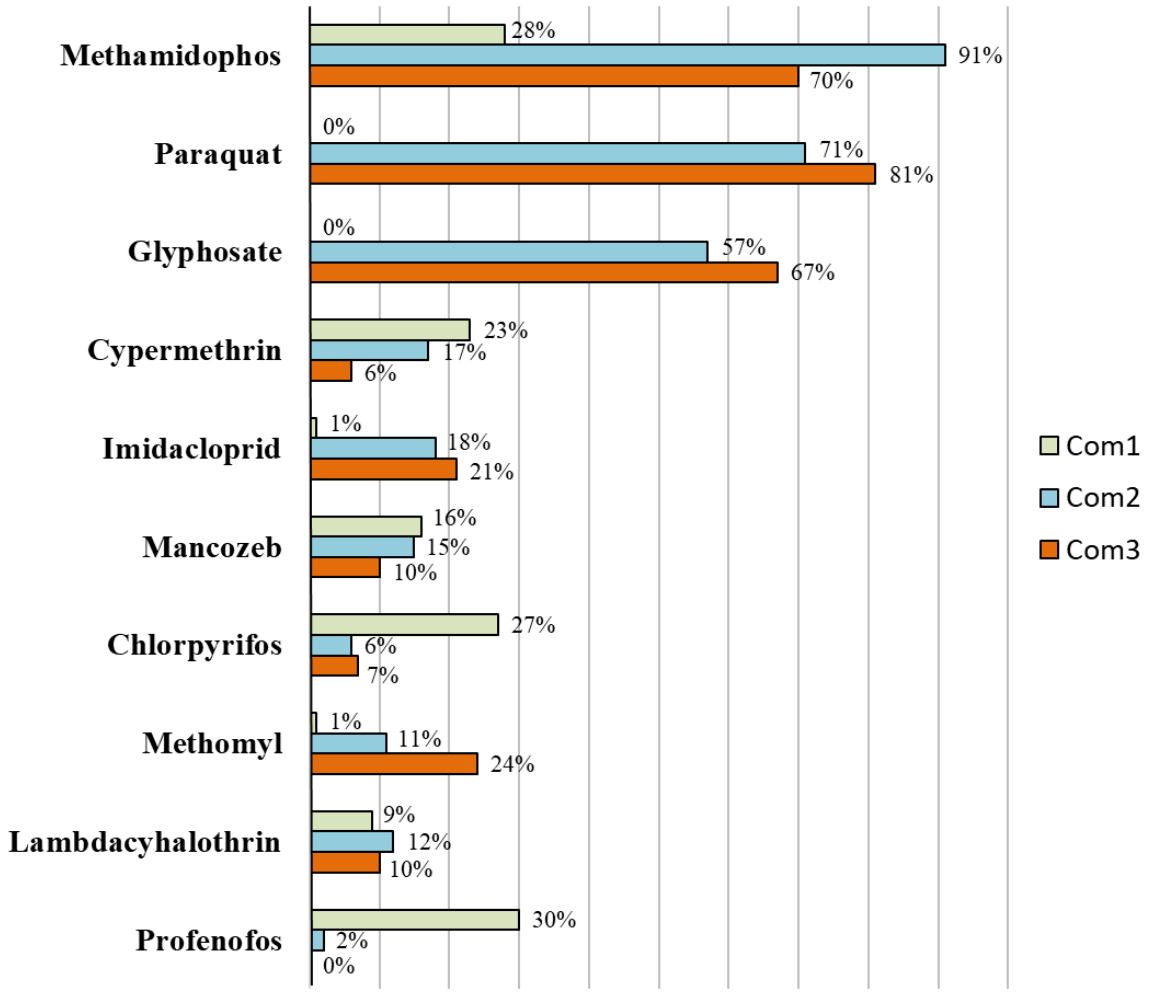

Figure 4. Some of the most commonly used pesticides in the three communities.

For applying pesticides, the FAO recommends that around 85% of the body should be covered with recommended PPE [52]. Here, only 17% of the farmers were well protected according to FAO criteria. Additionally, most of them only used one piece of clothing as PPE (41%), and most commonly a hat (76%) (Figure 5A). By comparison, women were less protected than men. Furthermore, only 59% of the farmers claimed to store the pesticides, PPE, and the rest of the equipment outside their houses.

Even though the FAO states: do not bury or burn pesticide containers as a disposal method [226], some pesticide traders and some Integrated Pest Management (IPM) trainers inform the farmers that burning small quantities of pesticide containers is allowed [73]. Consequently, burning was the most common way to get rid of the empty pesticide containers. It is well known that empty containers of pesticides abandoned in the environment can pollute the environment, implying a risk for human health [227]. Notably, 27% of the farmers stated that they throw the empty containers in the closest river, probably affecting the water quality (Figure 5B).

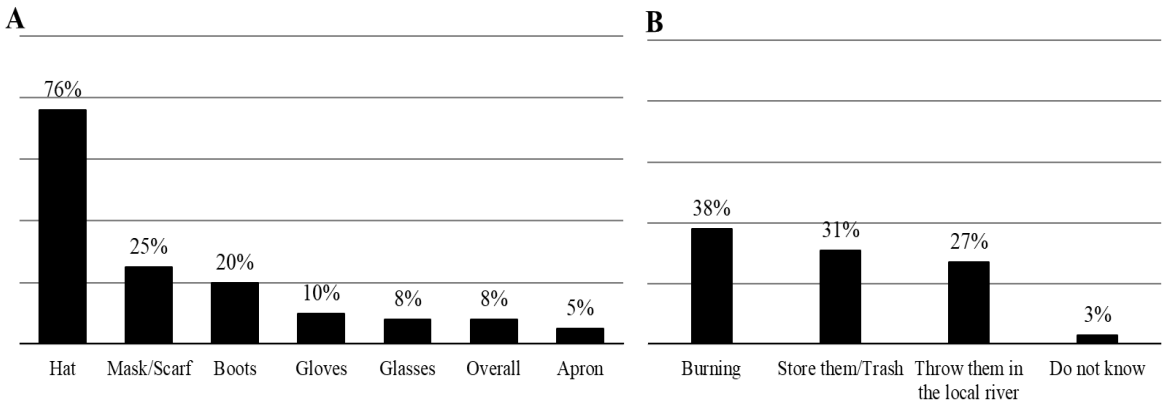

Figure 5. (A) Frequency of clothing as PPE. (B) Frequency of final disposal of pesticide containers.

4.1.3 Health effects related to exposure to pesticides

Since these are agricultural communities, crops grow close to the farmers' living areas. Consequently, 75% of the participants stated that they had felt pesticide odor around their houses. When asked if the farmers had experienced any health effects while or after they were applying pesticides, 80% of the farmers claimed to have been experienced one or several symptoms at least at one occasion, and especially women. More seriously, 52% reported having had at least three different symptoms that can be classified as acute pesticide poisoning. Headache was the most common, followed for burning eyes, dizziness, and red skin. Notably, women had experienced more APP symptoms than men, and especially in Com2. This finding was in accord with that only 4% of the women wore recommended PPE. Similar results and frequencies of APP were reported in Brazil, Spain, and northern Thailand, where neurological symptoms were the most common acute health effects after spraying pesticides [75, 228, 229]. Farmers in Thailand also reported a higher frequency of acute dermal effects compared with other symptoms [230]. These effects are expected when PPEs are not used as recommended but also depend on the type of pesticides that are applied.

Similar to other studies on women in farming communities [231, 232], against all the recommendations and probably due to lacking training, 31% of the women reported having sprayed pesticides while breastfeeding and 36% during pregnancy. Possibly, as a result, almost 50% of the women reported having had at least one miscarriage. These frequencies are much higher than the official reports from the Bolivian health authorities in 2015, which reported only 14% and 22% of miscarriages in La Paz and Cochabamba respectively [233]. Besides, 17% of them reported having delivered a child with a malformation/stillbirth.

4.1.4 Exposure assessment and relationship between PHI score and risk of high pesticide exposure

UPM analyses showed that the farmers were highly exposed to chlorpyrifos and 2,4-D. When the data were compared between men and women, significantly higher concentrations of pyrethroids metabolites (3PBA), 2,4-D, and pyrimethanil (OH-PYR) were found in men and for cyfluthrin (4F3PBA) and thiabendazole (5-OH-TBZ) in women. Farmers in Com3 showed the highest concentrations of 2,4-D, which agreed with what was reported about the use of this pesticide in this community. High levels of chlorpyrifos were also observed in people who had worked < 3 years as farmers in comparison with those who had worked for ≥ 8 years. This suggests that less experienced farmers may be more exposed to some pesticides while spraying compared to the more experienced farmers.

Farmers from Com1 showed a higher PHI score in comparison with the other two communities. Importantly, we found a reduction of risk of high pesticide exposure among farmers with a high PHI score for most of the pesticides. However, a significant protective effect was only shown for chlorpyrifos and cyfluthrin. This effect was also observed when the model was adjusted for gender and age. Likewise, in a study performed in Canadian farmers exposed to phenoxy herbicides, significantly lower urinary concentrations of 2,4-D were found because of the use of PPE in comparison with those farmers who did not use it [234]. However, in the same study, an association between higher urinary levels of MCPA and the use of complete PPE was found but in a very small number of farmers [234]. These results are also in agreement with what we found here with chlorpyrifos which rather indicated an increased risk of exposure among farmers who were better at following PPE recommendations. With these results, we confirmed the necessity of education and training in handling pesticides and the use of proper protection, not only for the Bolivian farmers but also for farmers in LMICs, in order to decrease exposure levels and harmful health effects produced by pesticides.

4.1.5 Influence of population characteristics and farming activities on levels of genotoxic damage

The results showed that women and participants over 42 years had higher levels of genotoxic damage. Age and gender were also correlated with high levels of DNA damage in other studies, concluding that those differences should be due to the DNA repair capability and differences in lifestyle factors [235, 236]. Men who were smokers showed higher levels of DNA strand breaks compared to female smokers. Although many studies showed an association between

alcohol consumption and a higher frequency of MN in peripheral lymphocytes [237], an opposite effect was found in this study. People who used to drink water from other sources but not from municipal water or water in bottles had higher levels of DNA strand breaks. This effect can probably be explained by the fact that some participants used to drink water from the river or underground water where many farmers claimed to dispose of the empty bottles or remains of pesticides [238].

Farmers from Com1 and Com3 were found to have higher levels of DNA strand breaks and MN frequencies compared with Com2. A possible explanation of that finding, and in agreement with other studies, could be that farmers in Com2 were younger compared with the other two communities, showing clearly the effect of the age and thus lifestyle factors and DNA repair capability [235, 236]. Also in agreement with other studies performed in non-farming populations [62, 239], our results showed that participants not actively spraying had similar levels of DNA damage to farmers who were spraying. Farmers who were actively working for 8 years or more showed a higher frequency of MN in comparison with farmers with fewer years actively working on the farm. This was most likely due to a higher age in the former group.

4.1.6 Associations between pesticide exposure and levels of genotoxic damage

The results revealed higher levels of DNA strand breaks in participants highly exposed to tebuconazole, 2,4-D, and cyfluthrin (UPM levels >75th percentile). Comparable outcomes were shown in farmers exposed to some OPs but in oral leukocytes and sperm of Polish and American men [240-242]. High exposure to cyfluthrin was also associated with a high frequency of NBUDs ($p < 0.001$). Additionally, similar results were found for tebuconazole and cyfluthrin in *in vitro* studies performed in peripheral lymphocytes [243, 244]. No other significant associations were found between genotoxic damage and high exposure levels for the other pesticides.

Logistic regression analysis between DNA strand breaks, MN frequency, and exposure levels was performed to assess the impact of high exposure to pesticides on the risk of having increased levels of genotoxic damage (> 75th percentile). The model was adjusted for age, gender, smoking, and alcohol consumption. The results showed a significantly increased risk for tail moment (OR = 1.99, CI: 1.10 – 3.60), and a borderline significant result for %DNA in tail (OR = 1.74, CI: 0.96 – 3.17) for 2,4-D. An increased risk of DNA strand breaks (OR > 1)

was also observed for tebuconazole and chlorpyrifos. This result was also found for 2,4-D and MN formation. In the recent classification on 2,4-D by IARC, it was concluded that oxidative stress is a likely important mechanism for the observed genotoxicity [245]. Although not studied here, the induction of oxidative stress was likely behind the strong observed correlation between exposure to 2,4-D and genotoxicity in our study. But we also have to consider that these farmers were not only exposed to one single compound but many other pesticides at the same time. The impact of exposure to mixtures is presented below.

Contrary to other researchers who showed genotoxic effects by pyrethroids in human peripheral blood lymphocytes [246, 247], here surprisingly, high exposure levels of pyrethroids (3-PBA) were associated with lower levels of DNA strand breaks. Similarly, high exposure levels of cypermethrin and permethrin (DCCA) was associated with a reduced risk of high levels of DNA damage (OR = 0.49 %DNA in tail; OR = 0.53 tail moment). The US EPA in 1988 [200], has classified cypermethrin in group C, as possible human carcinogen. Other studies have shown the genotoxic effect of cypermethrin in CHO cells and in organs and tissues of mice [248-250]. A systematic review of studies on cancer risk in humans from 2018 concluded that permethrin does not imply a risk of cancer in humans [251]. This protective effect found here for the pyrethroids must be interpreted with caution since the UPM most likely reflects an acute exposure, probably only from the day or days before the sampling. For that reason, more studies are needed to clarify this association.

4.1.7 Impact of exposure to pesticide mixtures on levels of genotoxic damage

The analysis of the exposure clusters showed that all clusters except cluster 3 displayed increased levels of DNA strand breaks compared to the control cluster 0. Participants included in cluster 7, which was mainly dominated by exposure to pyrethroids and one organophosphate (51% 4F3PBA, 27% of DCCA, and 18% TCP), displayed significantly higher levels of DNA strand breaks compared with cluster 0. This result was in accordance with the significant association that we found between cyfluthrin (4F3PBA) and DNA strand breaks. Since the logistic regression analysis for 2,4-D showed a significantly increased risk of genotoxic damage, it was not surprising that cluster 2, which was dominated by 2,4-D (80 %), showed an increased risk for genotoxic damage for DNA strand breaks (OR of 2.94, 95 % CI: 1.12–7.73). In contrast, all the clusters had similar or lower MN frequencies and with ORs ≤ 1, compared with cluster 0 (Figure 6). These results indicated a clear association between high exposure levels to some pesticides with increased DNA strand breaks but not with increased

chromosomal aberrations. Similar findings have been shown by other studies, suggesting that this effect could be a result of effective cellular repair mechanisms that respond to DNA damage (e.g. DNA strand breaks), reducing severe mutagenic effects such as micronuclei formation that can lead to malignant transformation of the cells [252, 253].

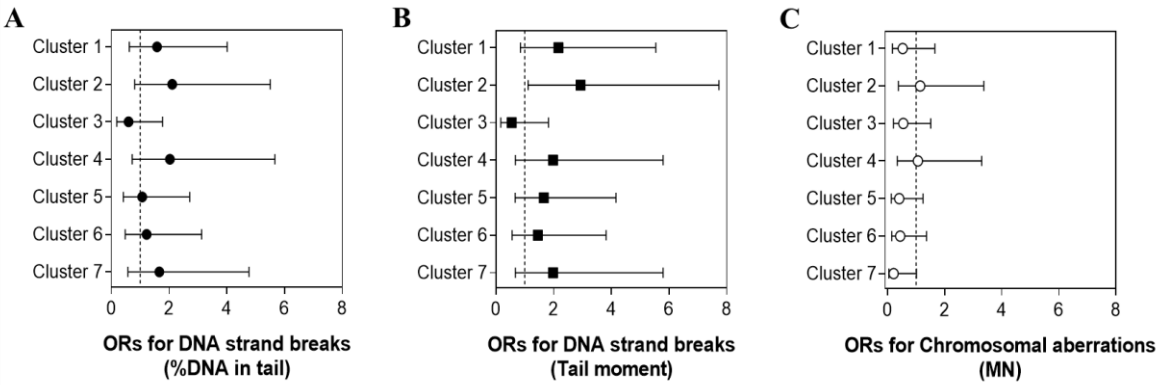

Figure 6. Associations between pesticide mixture exposures (by clusters) and risk of genotoxic damage. ORs for DNA strand breaks (A) %DNA in tail and (B) Tail moment. (C) ORs for chromosomal aberrations – Micronucleus.

4.1.8 Influence of GST genotypes on levels of genotoxic damage

Previous studies showed an association between an increased risk of genotoxic damage in farmers exposed to pesticides and null genotypes of GSTM1 and GSTT1 [149, 254, 255]. We found that 54% were GSTM1 null and 69% GSTT1 positive in our populations. Like other studies performed in occupational workers exposed to OPs and mixtures of pesticides in India [255, 256], higher levels of DNA strand breaks were found in individuals with GSTM1 null genotype than in those with GSTM1 positive genotype. Even though individuals with GSTT1 null genotype also had higher levels of DNA strand breaks, they were not statistically significant compared with GSTT1 positive. Moreover, the frequency of MN in both genotypes was higher in the positive individuals compared with the null genotypes, this was observed especially for GSTM1. This phenomenon was also found in a meta-analysis and other studies performed in South American countries and is probably due to the ability of GSTs to activate some chemicals into more reactive compounds [113, 148, 257]. Our results confirm that in order to reduce the risk of developing cancer, it is important to identify individuals who carry a GST null genotype, especially when they are occupationally exposed to environmental pollutants, such as pesticides [150].

4.2 *IN VITRO* STUDIES IN PAPER III

4.2.1 Induction of cytotoxicity and oxidative stress

The results showed a clear concentration-dependent reduction of HepG2 cell viability in response to most pesticides. Paraquat, methamidophos, and tebuconazole were the most potent pesticides with EC_{50} values of 0.25, 0.30, and 0.42 mM, respectively. Similar EC_{50} values were detected in other studies in HepG2 cells and other human cell lines for these three pesticides [258-260]. For the mixtures, the results showed that U3 was the only mixture that caused a significant reduction of cell viability and at its highest dose (74%, 1 mM). Since none of the mixtures caused a > 50% reduction of cell viability, EC_{50} values were not determined.

We found that the single and mixtures of pesticides neither affected the intracellular ROS induction nor transcription levels of genes involved in oxidative stress response (*SOD1*, *CAT1*, *GPX1*, and *HMOX1*). Even though paraquat previously has been used as a positive control to induce ROS formation [261, 262], it did not induce increased levels of ROS formation in this study. However, similar negative results were found in other studies when different human lung cancer cells were exposed to similar or lower concentrations of paraquat [259, 263]. Other studies, in human and animal cell lines, have shown that pesticide mixtures (e.g. different pyrethroids) may induce oxidative stress and/or neurotoxicity in a dose-dependent manner [264-266]. A remarkable finding here was that although we used concentrations below the EC_{50} for all the pesticides and mixtures, paraquat, profenofos, and mixture U3 caused ROS levels lower than what was observed for the DMSO control in response to their highest doses, which can suggest some type of cellular stress that was not detected with the viability assay.

Our results and the contradicting results found in published articles suggest that induction of ROS and oxidative stress in response to pesticides most likely depend on the time of exposure, concentration applied, and the type of cell model used and should thus be taken into consideration when planning *in vitro* experiments and interpreting results.

4.2.2 Activation of DNA damage signaling

Levels of pChk1 and γH2AX were determined 6 and 24 h after exposure, showing different time- and dose-dependent activations. At the earliest time point, the levels of pChk1 were increased by cypermethrin, paraquat, and tebuconazole. At the later time point, the levels of pChk1 were sustained for cypermethrin but increased up to 10-fold for paraquat in comparison with the control. The same effect was not found for tebuconazole, which seemed to provoke

only a transient activation of signaling. Also, at 24 h the highest concentration of profenofos (0.1 mM) induced high levels of Chk1 up to 5-fold. Very few studies have looked at the activation of Chk1 in response to pesticides. Huang et al. showed a concentration-dependent activation of Chk1 in response to the herbicide atrazine in normal human breast epithelial cells (MCF-10A) [267]. For γH2AX, all the pesticides caused a stronger induction at 24 h compared with the earlier time point. The induction of γH2AX was also stronger than for pChk1. At the late time point, paraquat and profenofos caused a concentration-dependent increase. Strong γH2AX induction has previously been shown in human lymphocytes exposed to glyphosate *in vitro* and in mammalian cells exposed to cypermethrin and paraquat [268-270]. In agreement with our observations, Huang et al. reported that activation of Chk1 by atrazine also was associated with increased levels of γH2AX [267]. Despite many *in vitro* studies have demonstrated the expression of γH2AX in human cells exposed to different xenobiotics [210], few studies have validated this biomarker in human populations. One study performed in an Indonesian population exposed to high natural radiation and other performed in Danish-twins aged 40 – 70 years reported a higher expression of γH2AX with non-significant differences or associations in gender or age [271, 272], concluding that more studies must be done for having better results and conclusions, On the other hand, *in vivo* studies performed in rats, have concluded that γH2AX is an ideal biomarker for genotoxicity testing [273, 274].

Since the single pesticides in general induced a stronger DNA damage response 24 h after exposure, all the mixture experiments were performed at this time point. The lowest concentration of mixture U2 and the highest concentration of S1 caused a significantly increased activation of pChk1. Similar to what we observed for the single pesticides, mixtures induced a stronger response for γH2AX compared with pChk1. γH2AX showed an evident dose-dependent increase in response to the mixtures U2, U3, and S1. We did not expect to have such a strong effect from mixture U3, since 2,4-D, which is the more abundant pesticide in this mixture (61%), did not induce γH2AX activation *in vitro* by itself. Other *in vitro* studies found that the combined effect of different pesticides can induce higher levels of γH2AX compared to single pesticides and probably due to the interaction effects of the pesticides [202, 275]. This could explain the strong effects observed here with mixture U3. Moreover, we found that the highest doses of paraquat (0.1 mM) and mixture S1 (0.2 mM) were the only exposures that induced gene expression of *CDKN1A* (Figure 7). These findings agree with the observed effects on pChk1 and γH2AX by the same compounds. Similar results for *CDKN1A* were found in a study performed on human lung A549 cells exposed to paraquat [259]. Together, these results suggest that even though some single pesticides are well-known genotoxicants, the combined effect of the pesticide mixtures may not always be predicted.

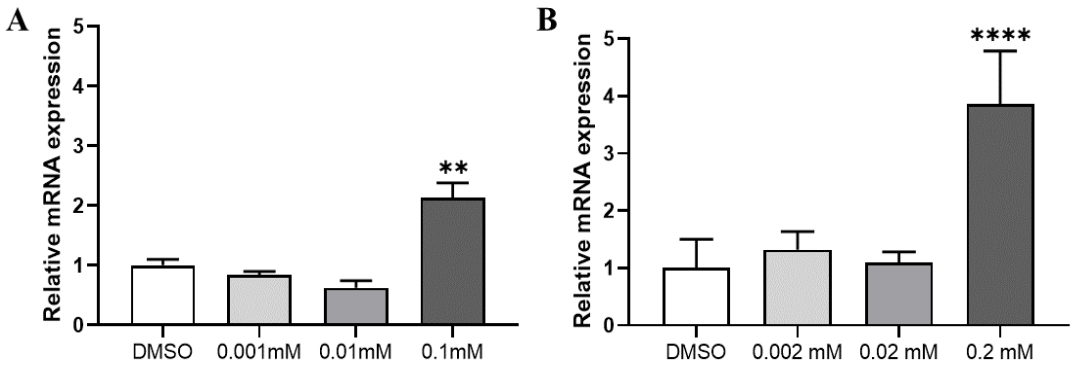

Figure 7. Gene expression analysis of *CDKN1A* at 24 h in response to (A) Paraquat and (B) mixture S1 (Data shows mean ±SE, n = 3) **p ≤ 0.01, p ≤ 0.0001.

4.2.3 Induction of genotoxic damage

Based on the DNA damage signaling results we tested paraquat, cypermethrin, tebuconazole, profenofos, and the 4 mixtures for their ability to induce DNA strand breaks. Single pesticides were tested at 6 and 24 h, and mixtures at 24 h. All the mixtures and the single pesticides induced a dose-dependent DNA damage increase (%DNA in the tail) at one or both time points. Paraquat was the most potent genotoxicant, its highest concentration induced up to 25-fold and 30-fold increase of DNA damage levels compared with DMSO at the two-time points, respectively (Figure 8). A significant increased effect was also caused by cypermethrin with its highest dose at the later point. Other *in vitro* and *in vivo* studies have reported that cypermethrin and profenofos can cause DNA and chromosomal damage [250, 276, 277]. Comparing the early with the later time point, DNA damage levels were either maintained or increased in response to all the pesticides but tebuconazole. This suggests that DNA damage induced by tebuconazole was more efficiently repaired in comparison with the other pesticides. This is further supported by our western blot results, which showed a transient activation of pChk1. In agreement, based on negative findings for cancer, genotoxicity, and mutagenicity *in vivo* and *in vitro* studies, tebuconazole was classified as non-genotoxic by the EFSA and WHO/IPCS [278, 279].

In agreement with what we observed for γH2AX in response to the mixtures, we observed a significant increase in the levels of DNA damage in response to the highest concentrations of mixtures U2 and S1 (Figure 8). The potent effect of S1 can probably be explained by the 25% of paraquat present in its composition since paraquat is considered as highly toxic and has classified as a possible carcinogen by the US EPA [200]. Moreover, *in vitro* and *in vivo* studies performed with paraquat have shown an induction of strand breaks and base modifications as

a consequence of oxidative damage to DNA [280, 281], Even though mixture U3 induced high levels of γH2AX, this was not associated with a significant DNA damage increase. Since mixture U1 did not show a strong activation of DNA damage signaling or induction of genotoxic damage, this mixture was considered as the least genotoxic of the mixtures.

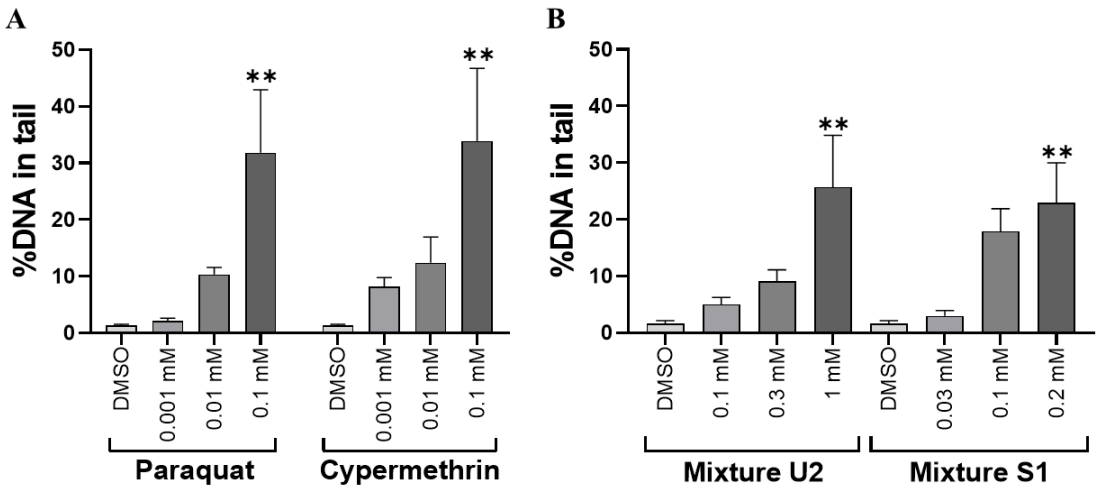

Figure 8. DNA damage at 24 h in response to (A) Paraquat and Cypermethrin and (B) mixtures U2 and S1 (Data shows mean ±SE, n = 3) **p ≤ 0.01.

In paper II we reported that farmers belonging to Com3 had the highest levels of DNA strand breaks and micronuclei in peripherical lymphocytes followed by farmers from Com1 and Com2. The contradictory *in vitro* results for mixture U3 that we found here, could probably be explained by the limited number of pesticides that were combined in the mixture *in vitro*, which was based on UPM detection and probably not reflecting the real exposure levels/composition. Another explanation could be the fact that we are comparing two different studies, one *in vitro* in human liver cells, where all the variables were under control, and the other *in vivo* on peripheral human lymphocytes from exposed farmers, where many uncontrolled variables could have interacted, thus affecting the results in a positive or negative way. However, these *in vitro* results may provide guidelines for continuing to investigate the different mechanisms involved in this field and to use them as a basis for future studies.

5 CONCLUSIONS

This thesis had the aim of biomonitoring the agricultural Bolivian population to assess if there was a correlation between exposure to pesticide mixtures and genotoxicity. Therefore, population and *in vitro* studies were performed in order to achieve this objective. The most important findings in this thesis can be divided into population and *in vitro* findings.

From our population results we can conclude that (i) the Bolivian agricultural community is highly exposed to mixtures of hazardous pesticides which could constitute a major health risk, (ii) the low use of PPE and the mishandling of pesticides constitute major determinants for high exposure to pesticides, and (iii) high exposure to some pesticides might cause an increased risk of genotoxic damage among Bolivian farmers. One additional crucial conclusion is the importance of education and training in the use of pesticides in LMICS such as Bolivia. In general, we found that farmers who were better at following recommendations for handling pesticides and the use of PPE had a significantly lower risk of being highly exposed to pesticides. Due to the necessity of a practical and easy to understand information about PPE and handling of pesticides, we designed a short brochure explaining these points. The brochure was distributed for free to all the participants of our study and to who was interested (see appendix).

Because both natural and artificial substances are mixed and interact in the environment, people are rarely exposed to single substances. Due to the possible combined action of these substances in the body, the toxic effects they may cause can be difficult to assess in populations. To mimic similar exposure scenarios as observed in populations, and to study possible mixtures effects, the use of *in vitro* studies is a good option.

Based on this, we carried out our *in vitro* studies, from which we can conclude that: (i) we confirmed and demonstrated the *in vitro* genotoxicity induced by common pesticides used in agricultural Bolivian populations and worldwide, (ii) the role of oxidative stress as a mechanism of DNA damage induction was not confirmed by our results, even though it was proved by other studies. This effect could be due to that we could not include all the pesticides that the population is really exposed to or the choice of cell model. (iii) Similar to our population study, we can conclude that some pesticides may act as drivers of toxic effects in our pesticide mixtures. This was clearly reflected in the mixture which contained paraquat in its composition, which was shown to be the most genotoxic mixture.

Besides these conclusions, I would like to emphasize that most of the studies about mixtures of pesticides and risk assessment approaches were based on the additivity effect, contrary, other studies have suggested the presence of a non-additive effects. Even though the additive or non-additive effects induced by mixtures were not studied in this thesis, our results can be used as a motivation for further studies.

Finally, the results of this thesis demonstrate that *in vitro* and population studies can be combined to support to each other in order to obtain a better understanding about the mechanisms of human health-effects from exposures to potentially harmful agents including mixtures of pesticides.

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