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Pioneering Insights: The Intersection of Pesticide Exposure, Genetic Variations, and Health Risks in Southeastern Brazilian Farmers

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Abstract: Brazil is the world leader in pesticide consumption, and its indiscriminate use puts farmers' health at risk. The *CYP2C9* gene stands out for encoding the *CYP2C9* enzyme, which metabolizes several endogenous substrates and specific xenobiotics, especially pesticides. Our goal is to study the risk of pesticide use, especially the herbicide glyphosate, in the development of diseases and the association with two *CYP2C9* polymorphisms, in farmers living in the southern region of Espírito Santo state, Brazil. The allelic frequency of *CYP2C9*1, CYP2C9*2* and *CYP2C9*3* was determined in blood samples from individuals exposed or not to pesticides using real-time PCR. 304 blood samples were analyzed, dividing *CYP2C9* genotypes into three metabolization classes: normal, intermediate, and slow. Our results show that normal metabolizers are more vulnerable to diseases such as blood pressure changes and cardiovascular and kidney problems, while intermediate metabolizers show an association with attention deficit disorder and miscarriages, suggesting that farmers in the studied region developed symptoms correlated to the *CYP2C9* genotype they have. This work is pioneering in associating specific genetic variations and health risks with pesticide exposure, emphasizing the importance of personalized medicine and stricter regulation of pesticide use for public health and occupational safety.

Keywords: *CYP2C9*; pesticides; glyphosate; cardiovascular disease; attention deficit; hypertension

1. Introduction

Brazil leads the world rank in pesticide consumption, a worrying fact that is directly related to the country's agricultural policy, focused on increasing productivity to compete in the international market [1]. Excessive use of pesticides, often without due technical information and without the adequate use of personal protective equipment (PPE), exposes farmers, their families and even the end consumer to significant health risks [2–5]. Substances widely used in agriculture, such as

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organophosphates, carbamates, malthion, diazinone, and especially glyphosate, have been classified as probable carcinogens by the International Agency for Research on Cancer [6–8]. The Cytochrome P450 family of enzymes, including *CYP2C9*, is essential for xenobiotic metabolism, having a characteristic molecular structure that allows the metabolization of several substances [9–11].

Recent studies have changed the perception about pesticide toxicity to humans, with evidence that substances such as glyphosate can inhibit the activity of the *CYP2C9* enzyme [12–14]. Furthermore, there is an association between central nervous system neoplasms and chemically exposed occupations, such as agriculture [15–17]. Symptoms of pesticide poisoning can range from acute effects, such as headaches and nausea, to chronic conditions, including liver and kidney damage [18,19].

In this study, the impact of pesticides on the development of diseases/symptoms in farmers of the south of Espírito Santo was evaluated, focusing on the relationship between daily exposure and *CYP2C9* polymorphisms and seeking to correlate the emergence or worsening of diseases with these polymorphisms, analyzing a total of 43 variables related to diseases and symptoms.

2. Materials and Methods

2.1. Study population

This research was submitted to the research ethics committee of the Federal University of Espírito Santo and approved according to opinion number 3.378.510 and the resolutions of the National Health Council nº 466 (of December 12, 2012) and nº 510 (of April 7, 2016). Inclusion criteria were men and women over 18 years of age, resident farmers of the south of the Espírito Santo state. A questionnaire was applied to each volunteer, and then blood was collected. DNA extraction and genotyping of 304 DNA samples were analyzed, subdivided into two groups: YES - Farmers who use pesticides (case/test) and NO - Farmers who do not use pesticides (control).

2.2. DNA extraction

DNA extraction and purification were carried out using a Qiagen® commercial kit, following manufacturer's recommendations (Qiagen, USA).

2.3. Real-Time PCR to Assess CYP2C9 Polymorphisms

CYP2C9 polymorphisms were identified by real-time PCR as previously described by Perini et al., (2009). Validated TaqMan® assays (Applied Biosystems, USA) were used to discriminate *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910) alleles. For both assays, real-time PCR reactions were performed in a final volume of 10 µl containing 30 ng of DNA, 1X Taqman Universal Master Mix (Applied Biosystems, USA), 1X of each specific assay and H2O q.s.p. PCR conditions were initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of denaturation at 92 °C for 15 seconds and annealing/extending at 60 °C for 1 minute. All samples were analyzed using the Fast 7500 Real-Time System (Applied Biosystems, USA). Genotypes were determined by analyzing the allelic discrimination plots. According to each sample's alleles, individuals were classified into different categories regarding their metabolization capacity:

Category	Metabolization	Genotype(s)
A	Normal	*1/*1
B	Intermediate	*1/*2; *1/*3
C	Slow	*2/*2; *2/*3; *3/*3

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It is important to emphasize the scarcity of cases in category C, making future analysis essential for possible connections with experiments not addressed in this study. In this context, we focused primarily on groups A and B, recognizing the need for comprehensive understanding to explore potential correlations and broader implications.

2.4. Data analysis methodology

For bivariate statistical analysis, the Chi-square test and, when necessary, Fisher's exact test was applied, with a 5% margin of error. To carry out the analyses, the free R version 3.6.1 software was used. The software can be obtained from the website https://cran.r-project.org/bin/windows/base/. Furthermore, all results derive from the bivariate analysis between "use of pesticides" and "appearance of a certain symptom" for each genotype group.

3. Results

Allelic and genotypic frequencies were calculated and presented in Tables 1 and 2.

Table 1. Genotypic frequencies (N=304).

Genotypes	n	Frequency
*1/*1	196	0.645
*1/*2	68	0.224
*1/*3	28	0.092
*2/*2	5	0.016
*2/*3	5	0.016
*3/*3	2	0.007

N: total number of individuals; n: sample subtotal; Frequency=n/N.

Table 2. - Allelic frequencies (N=608).

Allele	n	Frequency
*1	488	0.803
*2	83	0.137
*3	37	0.061

N: total number of individuals; n: sample subtotal; Frequency=n/N.

Most of interviewed workers (72.01%) were males and 27.99% were females. Farmers were predominantly aged between 34 and 50 years, having worked as such for 26 to 50 years, and 86 of them being in both groups (32.21%).

The percentage of rural workers who use pesticides was as high as 90.27%. Glyphosate was the most used (79.66%), followed by flutriafol (32.85%), thiamethoxam (26.91%) and cyproconazole (22.34%). Behavioral variables such as alcohol consumption and tobacco use were not correlated with genotypes and diseases/symptoms emergence.

Symptoms/diseases addressed were eye irritation, skin lesions/allergies, skin burns, nausea/vomiting, phlegm, abdominal pain, diarrhea, difficult digestion, wheezing, asthma, gastric inflammations, liver diseases, blood pressure changes, infertility, tearing, dizziness/vertigo, cough, shortness of breath/dyspnea, blurred vision, tremors, vomiting, joint pain, hepatitis, osteoarthritis/osteoporosis, kidney diseases, respiratory diseases, attention deficit, miscarriages, headache, excessive sweating, salivation, agitation/irritability, tingling, miosis, cramps, body/muscle pain, depression, cardiovascular diseases, cancer, heart palpitations, salivary gland damage, malformation and hyperactivity. Significant correlations ($P<0.05$) between the use of pesticides and symptoms/diseases were tested for each genotype group ("A", "B" and "C").

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As shown in Table 3, "B" intermediate metabolizers (who use pesticides) are associated with attention deficit (Chi-square P = 0.01782 / Fisher P = 0.02008) and miscarriages (Chi-square P = 0.00070 / Fisher P = 0.00409), and in normal "A" metabolizers (who use pesticides) an association with blood pressure alteration (Chi-square P = 0.00371 / Fisher P = 0.00305), cardiovascular diseases (Chi-square P = 0.04308 / Fisher P = 0.03319) and kidney diseases (Chi-square P = 0.06536 / Fisher P = 0.02635) was found.

Table 3. Diseases and symptoms of intermediate metabolizers "B" and normal metabolizers "A" of CYP2C9.

Disease/Symptoms	Group	P-value (Chi-square)	P-value (Fisher)
Blood pressure alterations	A	0.00371	0.00305
Cardiovascular diseases	A	0.04308	0.03319
Kidney diseases	A	0.06536	0.02635
Attention deficit	B	0.01782	0.02008
Miscarriages	B	0.00070	0.00409

4. Discussion

In this paper, we have investigated a possible link between exposure to glyphosate and other pesticides among agricultural workers in Brazil and various health issues and have explored specifically whether different polymorphisms of the *CYP2C9* liver enzyme might play a role in how pesticides impact health. We have found a statistically significant increase in blood pressure issues (hypotension/hypertension), cardiovascular disease, kidney disease, attention deficit, and miscarriages, when comparing pesticide-exposed agricultural workers with controls who worked on organic farms, with interesting differences observed between normal metabolizers (*1 alleles only) and intermediate metabolizers (*1 and either *2 or *3). In particular, normal metabolizers (group A) are more susceptible to hypertension, cardiovascular disease and kidney disease, whereas intermediate metabolizers (group B) are more susceptible to attention deficit and miscarriages. Sparse data prevented us from reaching any conclusions about group C (slow metabolizers), who had *2 and/or *3 alleles.

Glyphosate stands out as the most used pesticide by Espírito Santo farmers. According to Boocock et al. [20], glyphosate inhibits plant growth by interfering with the production of essential aromatic amino acids, primarily through suppression of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). In the terrestrial environment, glyphosate is mainly biodegraded into aminomethylphosphonic acid (AMPA). When metabolized by soil bacteria, glyphosate can cause several toxicological problems if absorbed. It can leak into groundwater and be transformed into formaldehyde, which is carcinogenic and neurotoxic [21]. Another problem with glyphosate-based herbicides is the presence of N-nitrosoglyphosate (NNG) as a contaminant (0.1 ppm), which is a highly carcinogenic substance [21]. NNG can also be formed in soil, water or in the human body when glyphosate combines with nitrates and nitrites [21].

Glyphosate is a glycine molecule with a methyl-phosphonyl group attached to the nitrogen atom. By acting as a glycine analogue, it is possible that glyphosate can displace glycine at random

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points during protein synthesis, with unknown consequences. Several synthetically produced amino acids, close structural analogues of natural amino acids, can be erroneously incorporated into peptides [22,23]. The correlations between glyphosate use and the recent alarming rise in several modern diseases are striking, as presented by Swanson et al. [24] These include obesity, diabetes, end-stage renal disease, kidney failure, autism, Alzheimer's disease, dementia, Parkinson's disease, multiple sclerosis, intestinal infection, inflammatory bowel disease, stroke, leukemia, thyroid cancer, liver cancer, pancreatic cancer and kidney cancer [24] .

We found a highly statistically significant correlation with pesticide use among Group A farmers for blood pressure alterations ($p = 0.003$). As early as 1974, hypertension had been identified as the most common and most potent contributor to cardiovascular mortality [25]. There is a 30-55% prevalence of hypertension in the general population in Europe, with higher rates in the elderly [26]. As of 2018, half the people in the United States over 20 years old suffers from hypertension [27]. A study based in China evaluated health risks of glyphosate exposure in factory workers involved in the production of glyphosate. Compared to workers at the same company who were not exposed to glyphosate, the exposed group had statistically significant higher rates of hypertension, coronary artery disease, elevated liver enzymes, and renal disease [28].

de Marins et al. [29], Ojelade et al. [30] and Gress et al. [31] have published literature reviews addressing potential cardiac issues associated with glyphosate exposure, and they found evidence of long QT syndrome, conduction blocks, arrhythmias, and cardiac arrest in cases of acute glyphosate exposure. These studies delved into the potential arrhythmogenic mechanism of glyphosate in mammalian cardiac tissues. Considering the rising use of synthetic molecules in agriculture, the review by Ojelade et al.[30] highlighted the adverse impact on human health and ecosystems [30]. Recently, the enzyme 21-hydroxylase, encoded by the CYP21A2 gene, was implicated in steroid hormone synthesis, suggesting that glyphosate's inhibition of CYP2C9 could impact endocrine regulation and influence farmers' blood pressure [32]. Glyphosate-based herbicides, widely used in woodlands and farmlands, have raised concerns due to toxicological issues of glyphosate and its metabolite AMPA in the food chain. Glyphosate has been linked to various health problems, necessitating a comprehensive review of its use, associated risks, and maximum residue limits. The compilation of such data aims to guide regulatory agencies in advising safe glyphosate usage practices [30].

Catani et al. [33] have shown that perinatal exposure of rats to glyphosate causes oxidative damage in the brain associated with reduced melatonin levels. Low urinary melatonin levels are associated with essential hypertension [34]. Melatonin supplementation in the evening has been shown to reduce nighttime blood pressure in men suffering from essential hypertension [35].

Gunatilake et al. [36] proposed that glyphosate, even without added formulators, has a unique insidious mechanism of toxicity that involves the erroneous substitution for the coding amino acid glycine during protein synthesis, which in certain circumstances can lead to nearly complete inactivation of the affected protein's enzymatic activity. Glyphosate's inhibition of cytochrome P450 enzymes in the liver would disrupt the liver's ability to detoxify and eliminate fat soluble toxic exposures, including pathogenic metabolites, toxic environmental chemicals, and prescription drugs [37], causing them to be much more nephrotoxic than they would normally be [38].

In this study, we observed that farmers classified as intermediate metabolizers (B) who used pesticides presented more "attention deficit" when compared to farmers who did not use pesticides. Although we did not find a direct correlation with this symptom in the literature, we observed that several researchers have found correlations between the use of pesticides and psychiatric diseases such as autism and Alzheimer's disease [39,40].

Attention deficit hyperactivity disorder (ADHD) has been conceptualized as a childhood disorder that diminishes with age. However, there is less awareness of adult ADHD as a condition, but a systematic study determined that 2.58% of the population worldwide continues to have symptoms of ADHD even after they age out of a childhood diagnosis, and, overall, 6.76% of the global adult population suffers from symptomatic adult ADHD [41]. A study of the trends over time in the United States of ADHD prevalence (according to US Centers for Disease Control and Prevention

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(CDC) data) showed a rising trend over time from 1990 to 2010, which correlated very strongly with the rising use of glyphosate on corn and soy crops ($R = 0.9466$, $p \leq 0.000036$) [42]. While correlation does not necessarily mean causation, there is an increasing number of peer-reviewed publications showing evidence that glyphosate causes specific pathologies that are linked with ADHD.

The incidence of ADHD in children is rising rapidly, from about 12 cases per 1000 people 30 years ago to about 35 cases per 1000 people in the late 1990s [43]. Learning and memory impairment are common ADHD symptoms. While ADHD has become very common in both children and adults, the causes of ADHD and the underlying brain pathology are still poorly understood. Recently, evidence is building to support the idea that ADHD is caused by an interaction between genetics and environmental factors linked to the Wnt and mammalian Target of Rapamycin (mTOR) signaling pathways, both of which are heavily involved in neurodevelopment [44]. Glyphosate exposure during pregnancy in rat dams caused suppression of the Wnt signaling pathway in exposed embryos, leading to behavioral and cognitive issues in the offspring [45]. Glyphosate exposure during pregnancy and lactation leads to abnormalities in the Wnt/β-catenin and Notch pathways in the prefrontal cortex of mouse offspring, potentially contributing to neurodevelopmental disorders [46].

Glyphosate has been linked to gut dysbiosis through suppression of critical beneficial microbes and overgrowth of pathogenic species. Pathogenic Clostridia species can produce toxic metabolites that cause neurological deviations in the brain, particularly when CYP enzymes are compromised [47]. Imbalances in the gut microbiome observed in association with ADHD could be related to impairments in dopaminergic signaling [48].

Glyphosate exposure in rats causes neurotoxicity by altering serotonergic, dopaminergic, and noradrenergic systems, with dose-related changes in neurotransmitter levels [49]. As early as 1999, dysregulation of central noradrenergic networks has been linked to ADHD [50]. These networks are involved in modulating high level cortical functions such as attention, alertness, vigilance, and executive function. Furthermore, disruptions in the dopaminergic system, particularly genetic defects in critical genes involved in dopamine signaling regulation, have been implicated in ADHD [51]. Genetic mutations involved in serotonin signaling have also been implicated in the etiology of ADHD [52]. In a machine learning model, serotonin transporter gene SNPs were linked to ADHD [53].

Arnsten et al. hypothesized in 2009 that a weakness in the prefrontal association cortex characterizes ADHD. This center is particularly dependent on dopaminergic stimulation and adrenergic stimulation for proper function. Stimulants that have been widely used to treat ADHD specifically enhance catecholamine signaling in the prefrontal cortex, increasing expression of both dopamine and norepinephrine [54].

Glyphosate has been shown experimentally to cause melatonin deficiency. Glyphosate exposure to rats prenatally and perinatally caused a 43% reduction in melatonin serum levels measured after pups had matured, likely through epigenetic effects [33]. The mechanism might be traced to the shikimate pathway, which glyphosate suppresses in gut microbiota. Melatonin is derived from the amino acid tryptophan, which is one of the three aromatic amino acids synthesized by gut microbes via the shikimate pathway. *In vitro* studies with rat pinealocytes exposed to 50 μm glyphosate showed that glyphosate activated metabotropic glutamate receptors, and that this caused a reduction in melatonin synthesis [33]. Metabotropic glutamate receptor activation suppresses melatonin synthesis in rat pinealocytes [55].

According to a review paper published in 2022, glyphosate exposure can cause many neurotoxic effects, affecting cell development, neurotransmission, and causing neuronal death, and behavioral and motor disorders in humans, rodents, fish, and invertebrates [56]. All these disruptions could adversely impact brain development, leading to symptoms of ADHD. Based on an extensive literature review, Seneff et al. [40] provided a theoretical argument for how glyphosate's suppression of melatonin synthesis and induction of oxidative stress in the brain through glutamate neuroexcitotoxicity could lead to the neurodevelopmental defects associated with autism. Glyphosate exposure to neurons disrupts synaptic assembly, and glyphosate reduces synaptic

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protein expression in the hippocampus, likely contributing to cognitive impairment observed in glyphosate-exposed developing rats [57].

Through genetic studies, deficiencies in melatonin signaling have been found in association with ADHD. In one experiment, 101 patients with ADHD were compared to 220 controls from the general population. Several damaging mutations were found in patients with ADHD. A specific mutation in N-acetylserotonin O-methyltransferase (ASMT) and another in melatonin receptor 1A (MTNR1A) were detected exclusively in ADHD patients, and both of these mutations were determined to abolish enzyme activity [58]. Melatonin therapy has been found to be beneficial for ADHD patients, particularly for treating insomnia [59].

One of the mechanisms involved in the emergence of these diseases is the ability of glyphosate to chelate metals. A study on Danish dairy cattle investigated the mineral composition in the serum of cattle fed feed containing traces of glyphosate in which cobalt (Co) and manganese (Mn) were deficient [14,60,61]. According to Samsel and Seneff [23], manganese (Mn) is one of the 14 essential trace elements in the human body, and its deficiency can explain pathologies associated with glyphosate use, notably autism and Alzheimer's disease, as well as attention deficit [62,63]. Another concern is that the glyphosate breakdown product AMPA can leak into groundwater and be transformed into formaldehyde, which is neurotoxic [21].

In this study, correlations were found between the use of pesticides and spontaneous abortions in group "B" of CYP2C9 genotypes (intermediate metabolizers). Swanson et al.[24] showed that the endocrine disrupting properties of glyphosate can lead to reproductive problems, such as infertility, miscarriage, birth defects and changes in sexual development. Glyphosate has also been linked to polycystic ovary syndrome, a major factor in female infertility, through its estrogenic properties [64]. Muñoz published a review paper showing that glyphosate has the key characteristics of an endocrine disruptor, based on its observed toxic effects [65].

Vianna-Jorge et al. [66] and Perini et al. [67] describe the frequencies of *1, *2 and *3 alleles in the Brazilian population, which was in accord with the frequencies we found in this study. Our results show that normal metabolizers "A" for *CYP2C9* (*1/*1) are more susceptible to developing a disease/symptom in relation to individuals carrying polymorphic variants of metabolizer groups "B" (*1/*2, *1/*3). To date, no metabolizing enzymes (including all CYPs) have been described to metabolize glyphosate in mammals. Secondary glyphosate metabolites generated by soil and intestinal bacteria, as well as glyphosate itself, have high toxicity [68]. The next step of this research should include the evaluation of CYP2C9 polymorphism in the general population to verify a correlation with symptoms/diseases observed in Espírito Santo farmers. Additionally, we would like to evaluate other diseases, particularly neurodevelopmental diseases such as autism, for their possible association with pesticides [47,62].

5. Conclusion

We have found that individuals who use pesticides and have *CYP2C9* "A" normal metabolizer (*1/*1) are more likely to develop diseases and symptoms compared to those who carry polymorphic variants "B" (*1/*2, *1/*3). We observed that Espírito Santo farmers exposed to pesticides manifested changes in blood pressure, cardiovascular and kidney diseases, miscarriages and attention deficit, correlating with their CYP2C9 genotypes. Continued research about the impacts of pesticide use on global health and the role of genetics in altering susceptibility will help guide new pesticide use policies.

6. Patents

Not applicable.

Supplementary Materials: Not applicable.

Author Contributions: Conceptualization, Débora Meira and Victor Kohls; Data curation, Débora Meira and Victor Kohls; Methodology, Débora Meira , Victor Kohls, Adriana da Silva and Jamila Alessandra Machado; Supervision, Iúri Louro; Validation, Adriana da Silva , Jamila Alessandra Machado and Iúri Louro;

Visualization, Raquel Trabach , Sonia Groisman, Elizeu de Carvalho, Stephanie Seneff and Iúri Louro; Writing – original draft, Débora Meira , Victor Kohls and Stephanie Seneff; Writing – review & editing, Matheus Casotti , Luana Louro, Gabriel Santana, Thomas Erik Louro, Lorena Altoé, Raquel Trabach , Sonia Groisman, Elizeu de Carvalho and Stephanie Seneff. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement Not applicable.

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Data Availability Statement: Our ethics committee asks that we not share sensitive participant data. We are willing to address any inquiries regarding the study methodology or results to the best of our ability. For further information, please contact Débora Dummer Meira (debora.dummer.meira@gmail.com).

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Conflicts of Interest: "The authors declare no conflicts of interest."

Additional research: NCBI. Results for variants in the *CYP2C9* gene. <https://www.ncbi.nlm.nih.gov/clinvar/?term=CYP2C9%5Bgene%5D>.

Abbreviations

ADHD: Attention deficit hyperactivity disorder; AMPA: aminomethylphosphonic acid; CDC: Centers for Disease Control and Prevention; Co: cobalt; EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase; Mn: manganese; mTOR: mammalian Target of Rapamycin; ASMT: N-acetylserotonin O-methyltransferase; MTNR1A: N-acetylserotonin O-methyltransferase receptor 1A; NNG: N-nitrosoglyphosate; PPE: personal Protection Equipment

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