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LUIZ PAULO DE AGUIAR MARCIANO

AVALIAÇÃO DO RISCO DA EXPOSIÇÃO HUMANA AOS FUNGICIDAS TRIAZÓIS NO SUL DE MINAS GERAIS: CONECTANDO BIOMONITORAMENTO E TOXICOLOGIA COMPUTACIONAL

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Ciências Farmacêuticas pela Universidade Federal de Alfenas. Área de concentração: Ciências Farmacêuticas.

Orientador: Profª. Drª. Isarita Martins
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O(A) Presidente da banca examinadora abaixo assina a aprovação da Tese apresentada como parte dos requisitos para a obtenção do título de Doutor em Ciências Farmacêuticas pela Universidade Federal de Alfenas. Área de concentração: Ciências Farmacêuticas

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— Milagres só acontecem para aqueles que não desistem!

(ODA, 2009, p. 6)

RESUMO

O Brasil se destaca como o maior produtor mundial de café, onde o uso extensivo de agrotóxicos é economicamente crítico, mas apresenta riscos à saúde e ao meio ambiente devido aos seus mecanismos de ação não seletivos. Especificamente, os fungicidas triazóis são amplamente utilizados na agricultura para gerenciar doenças fúngicas e são conhecidos por afetar enzimas CYP450 e microsomais do fígado em mamíferos. Esta pesquisa teve como objetivo realizar a caracterização do risco da exposição humana aos fungicidas triazóis por biomonitoramento de dose interna, medições de biomarcadores e integração de dados de *high-throughput screening* (HTS) através de fluxos de trabalho de toxicologia computacional utilizando o *Integrated Chemical Environment* (ICE). Voluntários da região sul de Minas Gerais, Brasil, foram divididos em dois grupos: trabalhadores agrícolas e cônjuges ocupacional e ambientalmente expostos aos agrotóxicos de áreas rurais (n = 140) e indivíduos da área urbana não expostos ocupacionalmente para servir como grupo de comparação (n = 50). Três fungicidas triazóis, ciproconazol, epoxiconazol e triadimenol, foram detectados nas amostras de urina de homens e mulheres do grupo rural por cromatografia gasosa acoplada à espectrometria de massas. Análise de biomarcadores indicou significativas alterações no grupo rural (Kruskal-Wallis $p < 0.0001$). Aumento na frequência de alterações celulares associadas a efeitos genotóxicos pelo ensaio do citoma de células de mucosa bucal e desequilíbrio oxidativo, particularmente entre homens expostos ocupacionalmente aos agrotóxicos. Além disso, elevação de ácidos biliares plasmáticos indicando alterações precoces e possivelmente reversíveis, pois não foram observadas diferenças significativas nos níveis de enzimas hepáticas (AST, ALT e γ-GT). Os hormônios androstenediona e testosterona foram significativamente reduzidos no grupo dos trabalhadores agrícolas (Mann-Whitney $p < 0.0001$). Os dados mostram uma associação inversa significativa de testosterona com colesterol, LDL, VLDL, triglicerídeos e glicose e uma associação direta com HDL (correlação de Spearman $p < 0.05$). No fluxo de trabalho do ICE, ensaios de HTS *in vitro* com bioatividade foram identificados para os três triazóis detectados e três outros ingredientes ativos das formulações de agrotóxicos. Os dados de HTS curados confirmam bioatividades predominantemente relacionadas ao metabolismo de hormônios esteroides, processos de estresse celular e enzimas CYP450 impactadas pela exposição ao fungicida em concentrações ocupacionais e

ambientais relevantes com base nos modelos de extrapolação *in vitro* para *in vivo* (IVIVE). Para avaliar o risco associado aos níveis de triazóis urinários, foram calculados a *Estimated Daily Intake* (EDI) e o *Hazard Quotient* (HQ). As maiores EDI foram observadas para epoxiconazol, variando de 0,47 a 6,31 µg/kg-pc/dia para homens e 0,49 a 8,77 µg/kg-pc/dia para mulheres no grupo exposto. Na pior das hipóteses, considerando o maior valor detectado de triazol urinário encontrado, o HQ calculado para o epoxiconazol foi de 2,1 para homens e 2,9 para mulheres. Esses resultados caracterizam o risco significativo para a saúde humana, particularmente pela alta frequência e intensidade da exposição ao epoxiconazol. Este estudo destaca o papel crítico do biomonitoramento e a utilidade das ferramentas computacionais na caracterização de risco à exposição aos agrotóxicos e na minimização dos efeitos prejudiciais à saúde resultantes da exposição crônica.

Palavras-chave: avaliação do risco; agrotóxicos; triazóis; biomonitoramento; toxicologia computacional.

ABSTRACT

Brazil stands as the world's leading coffee producer, where the extensive use of pesticides is economically critical yet poses health and environmental risks due to their non-selective mechanisms of action. Specifically, triazole fungicides are widely used in agriculture to manage fungal diseases and are known to disrupt mammalian CYP450 and liver microsomal enzymes. This research establishes a framework for risk characterization of human exposure to triazole fungicides by internal-dose biomonitoring, biomarker measurements, and integration of high-throughput screening (HTS) data via computational toxicology workflows from the Integrated Chemical Environment (ICE). Volunteers from the southern region of Minas Gerais, Brazil, were divided into two groups: farmworkers and spouses occupationally and environmentally exposed to pesticides from rural areas (n = 140) and urban area individuals not occupationally exposed to serve as a comparison group (n = 50). Three triazole fungicides, cyproconazole, epoxiconazole, and triadimenol, were detected in the urine samples of both men and women in the rural group by gas chromatography coupled to mass spectrometry. Biomarker analysis indicated significant alterations in the rural group (Kruskal-Wallis $p < 0.0001$). Increased frequency of cellular changes associated with genotoxic effects by the oral mucosa cell cytome assay and oxidative imbalance, particularly among men occupationally exposed to pesticides. Additionally, elevated plasma bile acids indicating early and possibly reversible changes, as no significant differences were observed in liver enzyme levels (AST, ALT, and γ-GT). The hormones androstenedione and testosterone were significantly reduced in the farmworker group (Mann-Whitney $p < 0.0001$). The data show a significant inverse association of testosterone with cholesterol, LDL, VLDL, triglycerides, and glucose and a direct association with HDL (Spearman's correlation $p < 0.05$). In the ICE workflow, active *in vitro* HTS assays were identified for the three measured triazoles and three other active ingredients from the pesticide formulations. The curated HTS data confirm bioactivities predominantly related to steroid hormone metabolism, cellular stress processes, and CYP450 enzymes impacted by fungicide exposure at occupationally and environmentally relevant concentrations based on the *in vitro* to *in vivo* extrapolation (IVIVE) models. To assess the risk associated with urinary triazole levels, Estimated Daily Intake (EDI) and Hazard Quotient (HQ) were calculated. The highest EDIs were observed for epoxiconazole, ranging from 0.47 to 6.31 μg/kg-bw/day for men and 0.49

to 8.77 μg/kg-bw/day for women in the exposed group. In the worst-case scenario, considering the highest detected urinary triazole value, the calculated HQ for epoxiconazole was 2.1 for men and 2.9 for women. These results characterize the potentially significant human health risk, particularly from the high frequency and intensity of exposure to epoxiconazole. This study showcases the critical role of biomonitoring and utility of computational tools in evaluating pesticide exposure and minimizing the adverse health effects of chronic exposure.

Keywords: risk assessment; pesticides; triazoles; biomonitoring; computational toxicology.

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LISTA DE ABREVIATURAS E SIGLAS

AC50	*Half-maximal activity concentration* ou metade da concentração de atividade máxima
ACC	*Activity concentration at the activity threshold cutoff* ou concentração de atividade no limite de corte de atividade
ADME	*Absorption*, *Distribution*, *Metabolism*, and *Excretion* ou Absorção, Distribuição, Metabolismo e Excreção
ALT	Alanina Aminotransferase
ANVISA	Agência Nacional de Vigilância Sanitária
AOEL	*Acceptable operator exposure level* ou nível aceitável de exposição do operador
AST	Aspartato aminotransferase
BSA	Proteína albumina do soro bovino
C	Concentração de triazol na urina (µg/g creatinina)
CA	Ácido cólico
CAAE	Certificado de Apresentação de Apreciação Ética
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Car	concentração no ar
CASRN	*Chemical identifiers* ou identificadores químicos
CAT	Catalase
CE	Valor de referência para excreção de creatinina em urina derivada de adultos no Brasil (1,22 g creatinina/dia)
CG-MS	Cromatografia gasosa acoplada a espectrometria de massas
cHTS	*Curated high-throughput screening* ou triagem de alto rendimento curados
CIP	Ciproconazol
COOMAP	Cooperativa Mista Agropecuária de Paraguaçu
CQA	Controle de qualidade alto
CQB	Controle de qualidade baixo
CQM	Controle de qualidade médio
DCA	Ácido deoxicólico
dp	Desvio padrão

DPR	Desvio padrão relativo
EAD	Equivalent Administered Dose ou Dose Administrada Equivalente
EC	Energia de colisão
EDI	*Estimated Daily Intake* ou Ingestão Diária Estimada
EDTA	Ácido etilenodiaminotetra-acético
EFSA	*European Food Safety Authority* ou Autoridade Europeia para a Segurança dos Alimentos
EMA	*European Medicines Agency* ou Agência Europeia de Medicamentos
EPA	*U.S. Environmental Protection Agency* ou Agência de Proteção Ambiental dos EUA
EPI	Equipamento de proteção individual
EPR	Erro padrão relativo
EPX	Epoxiconazol
ERE	Ensino remoto emergencial
ESI	Fonte de ionização por Electrospray
ET	Exposure time ou tempo de exposição
EUA	Estados Unidos da América
F	Fator de excreção urinária de triazol
FDA	*Food and Drug Administration*
GCA	Ácido glicocólico
GDCA	Ácido glicodesoxicólico
GPx	Glutationa peroxidase
HDL	*High density lipoprotein* ou Lipoproteína de alta densidade
HPLC	*High performance liquid chromatography* ou Cromatografia líquida de alta eficiência
HQ	*Hazard Quotient* ou Quociente de Risco
HTS	*High-throughput screening* ou Rastreio de alto rendimento
httk	*high throughput toxicokinetic* ou toxicocinética de alto rendimento
ICCVAM	*Interagency Coordinating Committee for the Validation of Alternative Methods* ou Comitê Interagências de Coordenação para a Validação de Métodos Alternativos
ICE	*Integrated Chemical Environment* ou Ambiente Químico Integrado
IDA	Ingestão Diária Aceitável

IR	*Inhalation rate* ou taxa de inalação
IVIVE	*in vitro* to *in vivo* extrapolation ou extrapolação *in vitro* para *in vivo*
LD	Limite de detecção
LDL	*Low density lipoprotein* ou Lipoproteína de baixa densidade
LIQ	Limite inferior de quantificação
m/z	Razão massa/carga
MDA	Malonaldeído
MET	Metconazol
MS/MS	Espectrômetro de massas em tandem
NAMs	*New Approach Methodologies* ou Novas Abordagens Metodológicas
NGRA	*Next generation risk assessment* ou avaliação do risco da próxima geração
NICEATM	*National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods* ou Centro Interagências do Programa Nacional de Toxicologia para a Avaliação de Métodos Toxicológicos Alternativos
NIEHS	*National Institute of Environmental Health*
NOAEL	*No observed adverse effect level* ou nível sem efeito adverso observado
P25	Percentil 25
P5	Percentil 5
P75	Percentil 75
P95	Percentil 95
pc	Peso corporal
PIB	Produto interno bruto
PROAP	Programa de Apoio à Pós-Graduação
PRP	Propiconazol
PTBK	*Physiologically based toxicokinetic* ou toxicocinética baseada fisiologicamente
R^2	Coeficiente de determinação
SCAN	Varredura
SEEM	*Systematic Empirical Evaluation of Models* ou Avaliação Empírica Sistemática de Modelos

SIM	*Single ion monitoration* ou monitoramento de íon selecionado
SOD	Superóxido Dismutase
TBARS	Ácido tiobarbitúrico
TCA	Ácido taurocólico
TCLE	Termo de Consentimento Livre e Esclarecida
TDCA	Ácido taurodeoxicólico
TDN	Triadimenol
TEB-D9	Tebuconazol-terc-butil-d9
UHPLC-MS/MS	*Ultra-high pressure liquid chromatography-tandem mass spectrometry* ou Cromatografia líquida de ultra pressão acoplado a espectrometria de massas em tandem
UNIFAL-MG	Universidade Federal de Alfenas
UV	Ultravioleta
UV-VIS	Ultravioleta visível
VALLME-CG/MS	Microextração líquido-líquido dispersiva assistida por vórtex
VLDL	*Very low density lipoprotein* ou lipoproteína de muito baixa densidade
γ-GT	Gama-glutamil transferase

SUMÁRIO

1 INTRODUÇÃO

A economia do Brasil é baseada no setor agrícola, uma vez que o país possui características climáticas favoráveis e uma grande quantidade de terras aráveis (Souza *et al.*, 2023). Atualmente, o Brasil é o maior produtor mundial de café, e essa comódite desempenhou um papel economicamente significativo na história do país (Souza e Navickiene, 2019). O estado de Minas Gerais possui o maior número de microrregiões, com destaque especial para o Sul de Minas Gerais, que se especializa em *Coffea arabica*, responsável por 75,4% da produção total de café e contribui com 81,4% do PIB do estado (Volsi *et al.*, 2019). No entanto, para sustentar e manter sua alta produtividade, o país depende do uso contínuo de agrotóxicos, e se tornou o maior importador de agrotóxicos do mundo na última década (FAO, 2023; Gonçalves e Delabona, 2022).

Os fungicidas triazóis representam o segundo maior grupo de agrotóxicos utilizados na região sul de Minas Gerais (Machado, 2018). O mecanismo geral de ação antifúngica dos triazóis envolve a inibição competitiva da CYP51 (lanosterol-14α-demetilase), que é uma enzima chave na biossíntese de esteróis em fungos (Giavini e Menegola, 2010; Tully *et al.*, 2006). No entanto, sua ação não é seletiva e também afeta os seres humanos, inibindo enzimas CYP450 e enzimas microssomais hepáticas em mamíferos, interferindo assim ativamente na produção de hormônios esteroides em humanos e outros danos à saúde (Giavini e Menegola, 2010; Machado *et al.*, 2021).

A avaliação do risco desempenha um papel vital como uma ferramenta importante e necessária que visa fornecer dados científicos confiáveis, garantindo a prevenção de efeitos maléficos à saúde humana (Carrão *et al.*, 2019). Para caracterizar o risco da exposição aos agrotóxicos, é fundamental considerar a análise da toxicidade dos compostos químicos, a compreensão das relações dose-resposta e a avaliação dos níveis de exposição. Biomarcadores de efeito são utilizados para avaliar o impacto desta exposição (Damalas e Eleftherohorinos, 2011).

Em relação à exposição aos agrotóxicos, é importante considerar fatores como a intensidade, frequência, duração da aplicação e métodos de segurança, incluindo o uso de equipamentos de proteção individual, além dos perfis físico-químicos e toxicológicos dos agrotóxicos utilizados. Por este motivo, é de extrema importância realizar o biomonitoramento de populações com potencial risco, através de

indicadores capazes de avaliar o grau da exposição (Machado e Martins, 2018). Estudos recentes têm adotado a "abordagem de dose interna" que envolve estimar a dose interna da substância no organismo (Fernández, Pardo, Adam-Cervera, *et al.*, 2020; Katsikantami *et al.*, 2019).

No entanto, na avaliação do risco, os pesquisadores e reguladores precisam ter acesso a dados confiáveis de toxicidade. Neste contexto, a toxicologia computacional refere-se ao uso de ferramentas computacionais para apoiar abordagens integrativas para pesquisas toxicológicas e avaliações de risco por meio de modelagem preditiva e análises complexas de dados para extrapolação e tradução entre fluxos de evidências, particularmente novas abordagens metodológicas (NAMs) baseadas na biologia humana que servem como alternativas aos testes em animais (Kleinstreuer, Tong e Tetko, 2020; Thomas *et al.*, 2019).

Assim, a avaliação do risco da próxima geração, denominada *Next Generation Risk Assessment* (NGRA), representa uma mudança em direção a avaliações baseadas na exposição e orientadas por hipóteses, prometendo avançar em avaliações de risco sem o uso de animais, que são mais rápidas e potencialmente mais relevantes para o ser humano (Dent *et al.*, 2021).

Portanto, este trabalho tem como objetivo a avaliação do risco da exposição humana aos fungicidas triazóis por biomonitoramento de dose interna, medições de biomarcadores e integração de dados de *high-throughput screening* (HTS) através de ferramentas de toxicologia computacional utilizando o *Integrated Chemical Environment* (ICE).

Realizou-se uma caracterização do risco da exposição humana aos fungicidas triazóis. O biomonitoramento de triazóis urinários utilizando o método de microextração líquido-líquido dispersiva assistida por vórtex de cromatografia gasosa acoplada à espectrometria de massa (VALLME-CG/MS), como indicador de dose interna, associado a um questionário para coletar dados sobre condições de exposição e características dos voluntários, para estimar o risco em relação aos fungicidas triazóis. Este estudo também avaliou biomarcadores de efeito *in vivo*, incluindo genotoxicidade, estresse oxidativo, ácidos biliares plasmáticos, enzimas hepáticas, e os hormônios androstenediona e testosterona, além de outros parâmetros bioquímicos. Acessamos dados *in vitro* de HTS para identificar perturbações de vias biológicas subcitotóxicas e alvos moleculares, e realizamos análises de extrapolação *in vitro* to *in vivo* (IVIVE) para comparar os dados de

biomonitoramento humano com modelos de exposição simulados para vias orais e inalatórias.

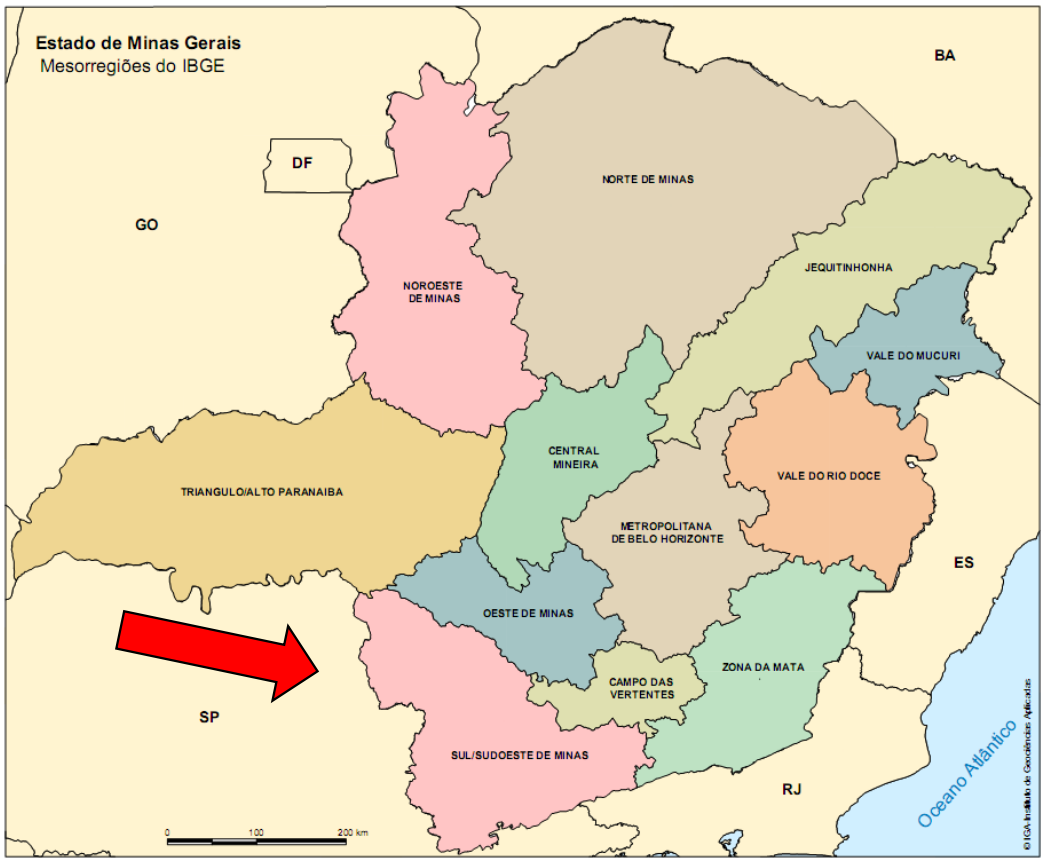
2 REFERENCIAL TEÓRICO

2.1 EXPOSIÇÃO AOS FUNGICIDAS TRIAZÓIS

A economia do Brasil é sustentada pelo setor agrícola, em virtude das características climáticas favoráveis e da vasta quantidade de terras aráveis disponíveis no país (Souza *et al.*, 2023). Atualmente, o Brasil é o maior produtor mundial de café, e essa commodite desempenhou um papel significativo na história do país (Souza e Navickiene, 2019). O estado de Minas Gerais, especialmente sua região sul, é conhecido por ser um dos principais cultivadores de *Coffea arabica*, sendo responsável por 75,4% da produção total de café e se tornou um pilar fundamental da economia de várias cidades no sul de Minas Gerais (Volsi *et al.*, 2019). Contudo, para manter e sustentar essa alta produtividade, o país tem dependido do uso contínuo de agrotóxicos, tornando-se o maior importador mundial desses produtos na última década (FAO 2023; Gonçalves e Delabona, 2022). A Figura 1 apresenta o mapa das mesorregiões do Estado de Minas Gerais.

Os agrotóxicos são substâncias químicas que atuam no controle de insetos, fungos, vermes e ervas daninhas que afetam as culturas na agricultura (Teodoro *et al.*, 2019). Embora haja benefícios em seu uso na agricultura, a exposição ocupacional, ambiental e dietética representa uma ameaça à saúde pública (Ye *et al.*, 2013). Os riscos ao ambiente e aos organismos decorrentes da exposição a esses produtos são influenciados por diversos fatores. Estes incluem o modo, a frequência, a quantidade e a concentração da aplicação que entra em contato, agravados pela falta de especificidade dos agrotóxicos ao alvo pretendido. A persistência de seus ingredientes ativos e produtos de degradação em diversos meios também exacerba esses riscos (Deknock *et al.*, 2019; Marsala *et al.*, 2020; Okoye *et al.*, 2022).

Figura 1 – Mapa das mesorregiões do Estado de Minas Gerais com destaque para a região Sul



Fonte: Adaptado de GAJO *et al.*, (2016).

A exposição ocupacional ocorre durante a produção, transporte, preparação e aplicação dessas substâncias químicas nos campos (Ye *et al.*, 2013). Os fatores que influenciam as exposições ocupacionais aos agrotóxicos incluem a intensidade e frequência da aplicação, a duração do contato, o método utilizado e práticas de segurança, como o uso de equipamento de proteção individual (EPI). E ainda deve ser considerado os perfis físico-químicos e toxicológicos dos agrotóxicos (Machado e Martins, 2018; Ye *et al.*, 2013). No entanto, observa-se frequentemente uma negligência no uso adequado dos EPIs e na sua higienização, o que aumenta significativamente o risco de exposição e intoxicação. Pesquisas indicam que, apesar da alta conscientização sobre a importância dos EPIs entre os agricultores, uma proporção substancial não os utiliza corretamente e alguns nunca os utilizam, ampliando o risco para si e para membros de suas famílias (Abreu e Alonzo, 2014; Pasiani *et al.*, 2012; Ye *et al.*, 2013).

No entanto, a prevalência dos agrotóxicos vai além dos ambientes

ocupacionais, expondo indivíduos também por vias ambientais. Isso inclui resíduos de agrotóxicos em alimentos cultivados localmente, água contaminada com resíduos de agrotóxicos, lavagem de roupas contaminadas durante aplicação, usos domésticos, aplicações ornamentais e deriva durante a aplicação (Knapke *et al.*, 2022).

Os fungicidas triazóis, que compõem 21% do mercado global de fungicidas, são utilizados na agricultura para controlar uma variedade de doenças fúngicas, como ferrugem, oídio e muitos fungos causadores de manchas foliares em frutas, vegetais, ornamentais e culturas de grãos (Cui *et al.*, 2021). São compostos heterocíclicos (fórmula molecular geral: $C_2H_3N_3$) e contêm um anel de cinco membros com dois átomos de carbono e três de nitrogênio (Souders *et al.*, 2019).

A atividade antifúngica dos triazóis deriva de seu mecanismo de ação para inibir competitivamente a CYP51 (lanosterol-14α-desmetilase), que é uma enzima chave na biossíntese de esteróis em fungos. A inibição da CYP51 causa a eliminação do ergosterol e o acúmulo de lanosterol e outros 14-metilesteróis, resultando em alterações nas paredes celulares fúngicas e consequente inibição do crescimento celular (Giavini e Menegola, 2010; Tully *et al.*, 2006; Xu *et al.*, 2020). Nesse contexto, os fungicidas triazóis são utilizado no combate à disseminação de pragas, destacando-se a ferrugem do cafeeiro, causada pelo fungo *Hemileia vastatrix* (Rhiney *et al.*, 2021).

No entanto, embora os triazóis inibam competitivamente a CYP51 em fungos, sua ação não é seletiva e também podendo impactar vias biológicas em mamíferos. Consequentemente, eles inibem CYP450 e enzimas microssomais hepáticas em mamíferos, resultando em alterações nos biomarcadores devido à exposição a esses compostos (Giavini e Menegola, 2010; Machado *et al.*, 2021). Esses grupos de fungicidas também exibem efeitos de disfunção endócrina, principalmente devido ao seu mecanismo de ação. Um efeito importante da inibição causada pelos triazóis está relacionado à CYP19 (aromatase), que pode contribuir negativamente para alterações na biossíntese de estrogênio em humanos. Essa monooxigenase catalisa a conversão de andrógeno em estrogênio por meio da clivagem do grupo metil no carbono 10 do anel esteroide de androstenediona e testosterona para produzir estrona e estradiol, respectivamente (Cui *et al.*, 2021; Knapke *et al.*, 2022).

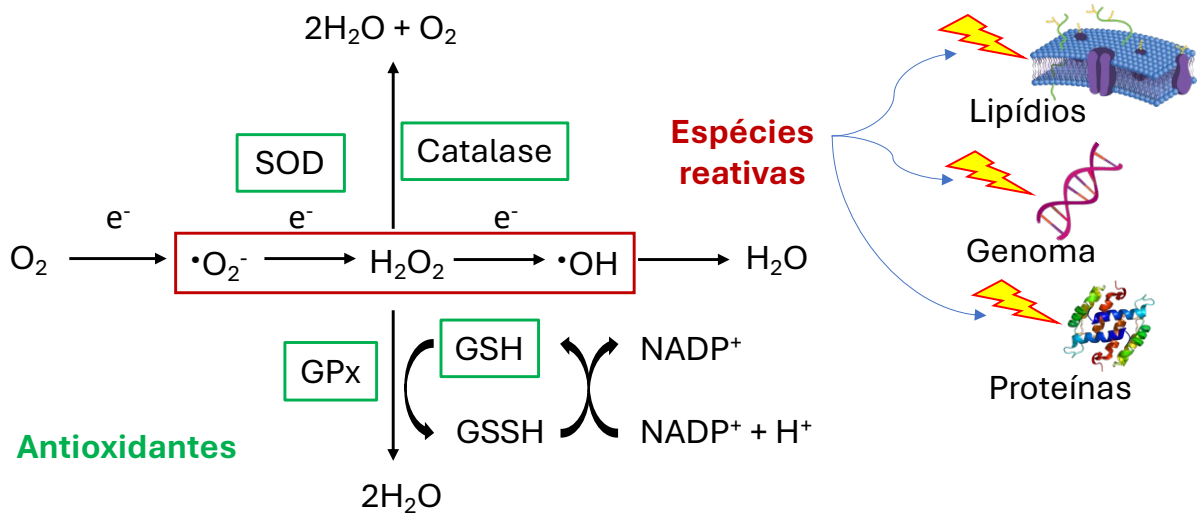
Além disso, estes compostos mostraram inibir a atividade da aromatase por meio de mecanismos competitivos (Heneweer, Berg e Sanderson, 2004). Possuem propriedades antiandrogênicas, evidenciadas pela inibição de receptores

androgênicos induzidos por testosterona, resultando na diminuição da secreção deste hormônio pelas células de Leydig (Roelofs *et al.*, 2014). Adicionalmente, estudos com cepas de leveduras que contêm plasmídeos com a sequência do receptor androgênico humano mostraram que os triazóis não apenas apresentam atividade antiandrogênica, mas também atuam como inibidores da CYP3A4, uma enzima importante no metabolismo de diversos xenobióticos e na metabolização da testosterona (Lv *et al.*, 2017). Embora os triazóis possam influenciar os receptores nucleares, sua ação parece ser mais proeminente na inibição das enzimas envolvidas na biossíntese de esteroides, com a CYP17 sendo particularmente afetada, onde a síntese de testosterona é inibida em concentrações inferiores àquelas necessárias para afetar a síntese de estradiol (Kjærstad *et al.*, 2010).

Outra área de estudo dos efeitos dos agrotóxicos é em relação ao estresse oxidativo. O estresse oxidativo é caracterizado por um desequilíbrio entre a produção de espécies reativas de oxigênio e a capacidade do sistema de defesas antioxidantes, resultando em danos a tecidos e componentes celulares (Simicic, Cudalbu e Pierzchala, 2022). Pesquisas indicam que os agrotóxicos podem perturbar esse equilíbrio, seja por meio da superprodução de espécies reativas ou pela alteração nos mecanismos de defesa antioxidante, o que inclui o aumento da peroxidação lipídica devido à interação das espécies reativas com membranas celulares (Jacobsen-Pereira *et al.*, 2018; Pisoschi e Pop, 2015). A Figura 2 apresenta o sistema antioxidante e ação de espécies reativas.

Para combater o estresse oxidativo, os organismos dispõem de enzimas antioxidantes como superóxido dismutase (SOD), catalase, glutationa peroxidase (GPx) e glutationa redutase (GSH-R), além das glutationas S-transferases (GST), que participam da detoxificação e fornecem defesa antioxidante. Estas enzimas são consideradas biomarcadores primários do estado antioxidante através de processos de oxidação e redução (Murakami, Nakabeppu e Sonoda, 2020; Oruç, 2010; Yang e Lee, 2015). Sob condições de estresse químico, a atividade do sistema de defesa antioxidante pode ser aumentada ou inibida. Embora a indução dessas enzimas seja uma característica adaptativa importante, em determinadas condições de estresse oxidativo, esse efeito pode também ser considerado como um mecanismo de adaptação significativo do organismo (Machado, 2018; Toni *et al.*, 2011).

Figura 2 – Sistema antioxidante e alvos de espécies reativas



Fonte: Adaptado de Murakami, Nakabeppu e Sonoda, (2020).

Os produtos de peroxidação lipídica, como o malondialdeído (MDA), servem como indicador comum para avaliar o estresse oxidativo, com o MDA sendo o aldeído mais abundante e considerado como um biomarcador de exposição (Ye, Liu e Li, 2016). Evidências de estresse oxidativo foram encontradas em peixes expostos ao propiconazol, com aumento da peroxidação lipídica e das proteínas oxidadas, além de uma redução nas respostas antioxidantes enzimáticas e não enzimáticas, indicando que a diminuição do GSH pode ser um processo regulatório para reestabelecer o equilíbrio oxidativo (Tabassum *et al.*, 2016). Similarmente, pesquisas com *Tetrahymena thermophila* expostas ao ciproconazol demonstraram uma resposta ao estresse oxidativo, marcada por uma redução na atividade da GST e nos níveis de GSH (Huang *et al.*, 2016).

E o desequilíbrio oxidativo pode desencadear efeitos genotóxicos (Cavallo *et al.*, 2021; Vecchiotti *et al.*, 2021). Estudos de biomonitoramento em indivíduos expostos aos agortoóxicos têm evidenciado a ocorrência de aberrações cromossômicas, trocas de cromátides irmãs e alterações nos perfis de micronúcleos, embora os resultados variem de acordo com o nível de exposição, o tipo de agrotóxico e misturas utilizados e as características geográficas e meteorológicas das áreas de aplicação (Costa *et al.*, 2023; Martínez-Valenzuela *et al.*, 2009; Silvério *et al.*, 2017).

O ensaio do citoma de células de mucosa bucal tem sido amplamente aplicado em estudos de avaliação de dano ao DNA devido a sua simplicidade e minimamente invasivo (Bolognesi *et al.*, 2015). Além disso, a utilização das células da mucosa bucal

permite investigar a capacidade regenerativa do tecido epitelial e o impacto de fatores como nutrição, estilo de vida, e exposição a substâncias genotóxicas que causam danos no DNA e morte celular (Thomas *et al.*, 2009).

A hepatotoxicidade é mais um dos efeitos tóxicos dos agrotóxicos e dos fungicidas triazóis (Ekman *et al.*, 2006; Goetz *et al.*, 2006; Heise *et al.*, 2018; Tully *et al.*, 2006). As lesões hepáticas podem ser classificadas principalmente em hepatocelulares, colestáticas, ou uma combinação de ambas. As lesões hepatocelulares são caracterizadas principalmente por afetarem os hepatócitos e frequentemente resultam em elevações das enzimas alanina aminotransferase (ALT) e aspartato aminotransferase (AST), com pouca ou nenhuma elevação da fosfatase alcalina. Já a colestase é diferenciada em intra-hepática, relacionada a fatores fisiológicos e patológicos dentro do fígado, e extra-hepática, que ocorre fora do fígado e pode ser causada por obstruções como cálculos biliares ou tumores. O diagnóstico dessas condições frequentemente utiliza níveis de fosfatase alcalina e ALT para determinar a natureza e extensão do dano hepático (Masubuchi *et al.*, 2016).

Consequentemente, os ácidos biliares séricos podem indicar a presença das doenças hepáticas e biliares. Os ácidos biliares são moléculas endócrinas que desempenham funções críticas além da absorção de nutrientes lipossolúveis, como a regulação de processos metabólicos importantes incluindo a homeostase de glicose e lipídios, e a excreção de substâncias tóxicas (Molinaro, Wahlström and Marschall, 2018; Vaz e Ferdinandusse, 2017).

A biossíntese ocorre no fígado e este processo sintetiza os dois principais ácidos biliares, o ácido cólico (CA) e o ácido chenodeoxicólico (CDCA), e é iniciado pela enzima CYP7A1. A enzima CYP8B1 é necessária para a síntese de CA, enquanto a CYP27A1, uma 27-esterol-hidroxilase mitocondrial, catalisa a oxidação da cadeia lateral do intermediário, contribuindo também para uma via alternativa que aumenta significativamente a produção total de ácidos biliares (Chiang, 2009; Vaz and Ferdinandusse, 2017).

Após a síntese, a maioria dos ácidos biliares é conjugada com glicina ou taurina pela enzima ácido biliar-CoA: aminoácido N-aciltransferase (BACAT), o que diminui sua toxicidade e aumenta a solubilidade para secreção na bile (O'Byrne *et al.*, 2003). Após a alimentação, esses ácidos são liberados no intestino, onde são modificados por enzimas bacterianas, resultando em ácidos biliares secundários como o ácido deoxicólico (DCA) e o ácido litocólico. A maior parte desses ácidos é reabsorvida e

recirculada, completando a circulação enterohepática e mantendo baixas concentrações fora deste sistema em condições normais (Chiang, 2009; Vaz e Ferdinandusse, 2017).

2.2 AVALIAÇÃO DO RISCO

As práticas de avaliação do risco regulatórias são essenciais para assegurar a proteção da saúde pública e ambiental. As agências governamentais têm a responsabilidade de revisar, quantificar e regular substâncias químicas, agentes físicos e farmacêuticos para prevenir danos potenciais. O *National Research Council* (NRC) em 1983 foi o primeiro documento que delineou um processo de quatro etapas para avaliação do risco (NRC, 1983; Stackelberg e Williams, 2021). Este processo multifacetado envolve uma abordagem metódica e disciplinada que engloba a identificação de perigos, a avaliação de dose-resposta, a avaliação de exposição e a caracterização de risco (Varshavsky *et al.*, 2023). Esses passos formam a espinha dorsal das práticas de avaliação do risco, integrando diversas disciplinas da toxicologia para fornecer uma análise tanto qualitativa quanto quantitativa dos riscos potenciais (NRC, 2007).

A identificação de perigos busca determinar os possíveis efeitos tóxicos à saúde associados a uma exposição química, enquanto a avaliação de dose-resposta estabelece a correlação entre a quantidade de exposição e a severidade dos efeitos. A avaliação de exposição quantifica o nível e a duração da exposição a que os indivíduos estão sujeitos, e a caracterização de risco sintetiza todas essas informações para descrever a natureza e a magnitude do risco à saúde. Este processo rigoroso assegura que as medidas regulatórias tomadas sejam baseadas em evidências científicas sólidas e reflitam as necessidades de proteção à saúde pública e ambiental (NRC 1983, 1994, 2007). As quatro etapas da avaliação do risco são:

a) **Identificação do perigo**: identificar as substâncias químicas suspeitas de apresentar riscos à saúde. Utiliza-se de dados de experimentação animal, ensaios *in vitro* e dados humanos de estudos epidemiológicos sobre o composto químico e suas propriedades físico-químicas e sua interação com o organismo humano e seus possíveis efeitos tóxicos.
b) **Relação dose-resposta**: avaliação mais aprofundada das condições sob

as quais as propriedades tóxicas de uma substância química podem se manifestar em pessoas ou animais expostas, com ênfase particular na relação quantitativa entre a dose e a resposta estudada. O desenvolvimento dessa relação pode envolver o uso de modelos matemáticos. Este passo pode incluir uma avaliação das variações na resposta, por exemplo, diferenças na susceptibilidade entre pessoas jovens e idosas.

c) **Avaliação de exposição:** especificação da população que pode estar exposta ao agente em questão, identificação das vias pelas quais a exposição pode ocorrer e estimativa da magnitude, duração e momento das doses como resultado de sua exposição.
d) **Caracterização de risco:** integração das informações dos três primeiros passos para desenvolver uma estimativa da probabilidade de que qualquer um dos perigos associados ao agente em questão ocorra em pessoas expostas. Os dados devem ser compilados e utilizados pelas autoridades reguladoras para tomar decisões informadas em questões de saúde pública.

Diferentes técnicas analíticas são utilizadas em avaliações do risco de câncer e de não câncer para quantificar o risco de uma substância química. O resultado final de uma avaliação do risco não cancerígeno pode ser a determinação de uma dose de referência humana quantitativa (RfD) para exposições orais ou de uma concentração de referência (RfC) para exposições por inalação (Barnes *et al.*, 1988; EPA, 2024). A RfD é a estimativa da dose média diária de compostos exógenos, que é comumente definida como a quantidade de uma substância química ao qual uma pessoa pode ser exposta diariamente por um longo período de tempo (geralmente uma vida inteira) sem sofrer um efeito deletério. É um componente importante da caracterização de risco de substâncias químicas (Men *et al.*, 2023). Ao desenvolver um valor de referência não cancerígeno (RfD ou RfC) para uma substância química, os órgãos regulatórios consultam a literatura científica e estudos subsidiados por empresas que solicitam novos registros e seleciona um efeito crítico (EPA, 2024; Hays and Kirman, 2017).

O efeito crítico, ou *point of departure* (POD) é definido como o efeito estudado, ou seu precursor conhecido, que ocorre na dose mais baixa identificada na espécie

mais sensível à medida que a dose aumenta no estudo da dose-resposta. Quando um nível sem efeito observado (NOAEL) pode ser identificado em um estudo crítico, ele se torna a base para a derivação do valor de referência regulatório (NRC, 2007). Se o NOAEL não puder ser identificado, então um nível de efeito observado na menor concentração (LOAEL) é utilizado em seu lugar. Recentemente, doses de referência (BMDs) da modelagem de dados de dose-resposta têm sido usadas em vez da abordagem tradicional NOAEL/LOAEL; no entanto, a maioria dos RfDs é baseada em NOAELs (EFSA *et al.*, 2017; EPA, 2024).

O NOAEL, LOAEL ou BMD é dividido por um fator de incerteza apropriado para derivar o valor de referência final. Os fatores de incerteza são geralmente de 10 vezes (mas podem ser maiores ou menores se informados por dados pertinentes) e destinam-se a contabilizar a incerteza nos dados disponíveis de variação na população humana, extrapolação de dados de animais para humanos, extrapolação de exposições menores que a vida útil para exposições ao longo da vida, extrapolação de um LOAEL em vez de um NOAEL, ou uso de bases de dados incompletas (Alexeeff *et al.*, 2002; Johanson, Moto e Schenk, 2023; NRC, 2007).

Curvas de dose-resposta são tradicionalmente construídas a partir de estudos em animais (Holsapple e Wallace, 2008). Para estimar os riscos abaixo dos níveis testados, os dados observados são usados para derivar um *point of departure* seguido por extrapolação para exposições mais baixas (EPA, 2005; NRC, 2007). Abordagens lineares ou não lineares podem ser usadas para extrapolar para doses baixas, e a escolha dos métodos é crítica porque as estimativas de risco derivadas variam de acordo com a técnica. Em geral, abordagens lineares produzem estimativas de risco mais conservadoras do que abordagens não lineares (EPA, 2024; Guérard *et al.*, 2015; NRC, 2006, 2007).

Portanto, a avaliação do risco devido à exposição a substâncias químicas requer informações sobre os mecanismos de ação, toxicocinética, relação dose-resposta e as vias de exposição. Os perigos são identificados, independentemente da potência toxicológica dos seus efeitos, e usados para atribuir um composto a uma categoria de classificação de perigo com base no Sistema Globalmente Harmonizado (GHS) ou em outro sistema de classificação. Embora a metodologia e as abordagens de avaliação do risco possam diferir em detalhes entre diferentes regiões ou órgãos regulatórios, elas são geralmente consistentes (Greim, 2001; Krewski *et al.*, 2010; Sewell *et al.*, 2021). Consequentemente, avalia-se o potencial da substância química

em causar efeitos prejudiciais à saúde, em que nível, duração e frequência de exposição, além de estimar a probabilidade de que esses efeitos ocorram. O processo é baseado na consideração da toxicidade intrínseca das substâncias químicas, geralmente determinada por testes de toxicidade animal especificamente projetados ou por meio de dados humanos de estudos epidemiológicos, e em exposições humanas preditivas ou medidas, integrando assim os estudos de dose-resposta e modo de ação (Dekant e Colnot, 2013; Dourson *et al.*, 2013).

Na avaliação do risco a exposição aos agrotóxicos em seres humanos, é levado também consideração, além do estudo da toxicidade, perfis toxicocinéticos e toxicodinâmicos, a exposição em si, através da aplicação de biomarcadores de dose-interna ou do ambiente, de efeito e também de susceptibilidade (Bonvallot *et al.*, 2021). As condições da exposição como modo de preparo da formulação, presença de adjuvantes, condições ambientais no momento da aplicação (a temperatura, umidade e presença de ventos), uso inadequado de equipamentos de proteção e a frequência de exposição são fatores que afetam significativamente o risco da exposição (Machado e Martins, 2018).

O biomonitoramento humano avalia estes biomarcadores que determinam concentrações internas das substâncias químicas ou seus metabólitos em amostras biológicas humanas, como urina, sangue, plasma, ou cabelo, bem como qualquer alteração bioquímica precoce (Fowler, 2012). Os biomarcadores podem ser classificados em biomarcadores de exposição, efeito ou suscetibilidade. Representam potenciais *endpoints* biológicos ou moleculares que são mensuráveis e que correlacionam, direta ou indiretamente, alterações celulares a exposições a produtos químicos ou medicamentos, ou ainda fases dos processos patológicos (Franco, Nardocci e Günther, 2008; Machado e Martins, 2018). O biomonitoramento agrega exposições de diferentes fontes e por diferentes vias, fornecendo, assim, uma estimativa mais precisa da carga corporal. A inclusão de dados de biomonitoramento pode, portanto, melhorar a precisão dos estudos de avaliação do risco tanto para a população em geral quanto para os trabalhadores, seja de forma separada ou como parte da população (Louro *et al.*, 2019).

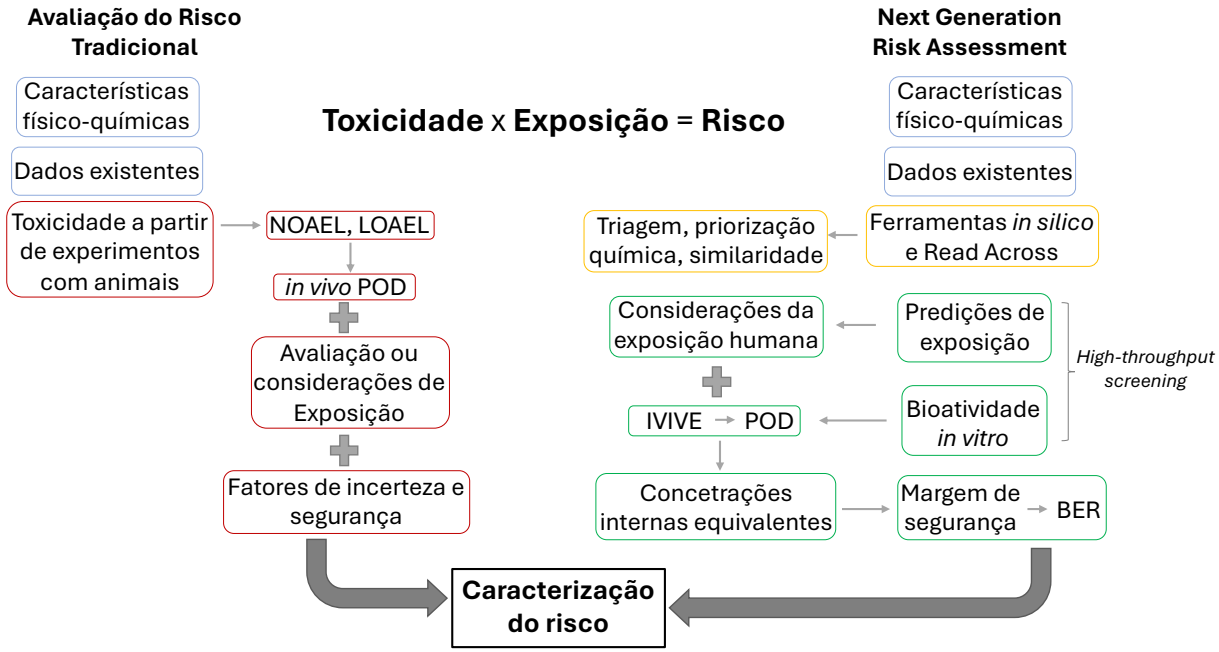
Na saúde ocupacional, o biomonitoramento tem o objetivo de identificar e controlar exposições em locais de trabalho e, quando disponível, comparar valores de limite biológicos estabelecidos para exposição ocupacional (Brasil, 2022; Huber *et al.*, 2022).

2.3 TOXICOLOGIA COMPUTACIONAL

É importante destacar que, na avaliação do risco da exposição aos agrotóxicos em humanos, fatores essenciais devem ser incluídos como a análise da toxicidade dos compostos químicos, no entendimento das relações dose-resposta e na avaliação dos níveis de exposição. Biomarcadores de efeito são utilizados para avaliar o impacto dessa exposição (Damalas e Eleftherohorinos, 2011; Machado e Martins, 2018). Consequentemente, nas duas primeiras etapas da avaliação do risco, pesquisadores e reguladores precisam ter acesso a dados de toxicidade confiáveis. No entanto, o enorme volume de dezenas de milhares de substâncias químicas que necessitam de avaliação toxicológica apresenta um enorme desafio. A praticidade de avaliar cada composto químico novo e existente por meio de estudos convencionais de toxicidade aguda e crônica em mamíferos é limitada por esses desafios (Hamm *et al.*, 2017; Mansouri *et al.*, 2021).

Neste contexto, a toxicologia computacional refere-se ao uso de ferramentas computacionais para apoiar abordagens integrativas para pesquisas toxicológicas e avaliações de riscos químicos por meio de modelagem preditiva e análises complexas de dados para extrapolação de resultados e predições, particularmente novas metodologias de abordagem (NAMs) baseadas na biologia humana que servem como alternativas aos testes em animais (Kleinstreuer, Tong e Tetko, 2020; Thomas *et al.*, 2019). Assim, a avaliação do risco da próxima geração, ou *next generation risk assessment* (NGRA) representa uma mudança em direção a avaliações baseadas na exposição e orientada por hipóteses, prometendo avançar avaliações do risco sem animais e que são mais rápidas e potencialmente mais relevantes para o ser humano (Dent *et al.*, 2021). A Figura 3 esquematiza as semelhanças e diferenças entre avaliação do risco tradicional e NGRA.

Figura 3 – Avaliação do risco tradicional e *next generation risk assessment* (NGRA)



Fonte: Do Autor.
Nota: POD: *point of departure*; IVIVE: extrapolação *in vitro* para *in vivo*; BER: *bioactivity exposure ratio*.

Essas abordagens adotam diversas formas, por exemplo, coletando diferentes tipos de dados experimentais em grande escala e aplicando modelos de *machine learning*, ou construindo representações matemáticas de sistemas biológicos e suas respostas a determinadas perturbações. A toxicologia computacional também é útil para fornecer modelos mecanicistas que avaliam a toxicidade de compostos químicos de maneira experimental e alto rendimento (Carlson *et al.*, 2022; Kleinstreuer, Tong e Tetko, 2020).

A colaboração de pesquisa federal dos EUA denominada Tox21 envolve esforços de agências como o *National Institutes of Health* (NIH), da *Food and Drug Administration* (FDA), do *National Toxicology Program* (NTP), e do progrma ToxCast da *Environmental Protection Agency* (EPA), e está está na vanguarda das práticas evolutivas de toxicologia para o século 21 (Carlson *et al.*, 2022). Esta colaboração dedica-se em avaliar de forma eficiente uma variedade de substâncias, incluindo substâncias químicas, agrotóxicos, produtos médicos e NAMs (Carlson *et al.*, 2022; Thomas *et al.*, 2018).

NAMs não são necessariamente métodos recém desenvolvidos. Sua inovação reside na aplicação em processos de tomada de decisão regulatória ou como substitutos ou complementos para os de testes de toxicidade tradicionais (Zalm *et al.*, 2022). Essas abordagens englobam estratégias de alto rendimento, computacionais

e são baseadas em biologia humana, desempenhando um papel crucial no desenvolvimento de modelos preditivos. Ao aproveitar os dados de triagem de alto rendimento, denominado dados de *high-throughput screening* (HTS) do Tox21 e do programa ToxCast, esses modelos podem ser instrumentais na delineação de perfis de bioatividade mecanicista relacionados a resultados toxicológicos (Browne *et al.*, 2018; Krishna, Berridge e Kleinstreuer, 2021).

Para promover ainda mais a interpretação e aplicação de dados HTS e fluxos de trabalho, também conhecidos como *workflows* de toxicologia computacional, o *National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods* (NICEATM) desenvolveu o *Integrated Chemical Environment* (ICE) (https://ice.ntp.niehs.nih.gov/). A plataforma ICE é fundamental para melhorar a acessibilidade e a relevância contextual de dados curados de alta qualidade, incluindo dados HTS (Abedini *et al.*, 2021; Daniel *et al.*, 2022; Hines *et al.*, 2022)

A ferramenta *Curve Surfer* do ICE fornece curvas dose ou concentração-resposta interativas de ensaios *in vitro* de HTS, extraídas da versão 3.5 do invitrodb da EPA (setembro de 2022). A ferramenta suporta análise e extração de valores como a concentração na atividade máxima de 50% (AC50), concentração de atividade no limite de corte de atividade, ou *activity concentration at the activity threshold cutoff* (ACC) e topo da curva (Abedini *et al.*, 2021). Os dados HTS curados, denominados cHTS na plataforma, também integram detalhes de controle de resultados de química analítica e sinalizadores para destacar dados potencialmente de baixa qualidade, assim fornecendo um entendimento biológico enriquecedor ao vincular ensaios in vitro e alvos mecanicistas e modos de ação toxicológicos (Daniel *et al.*, 2022).

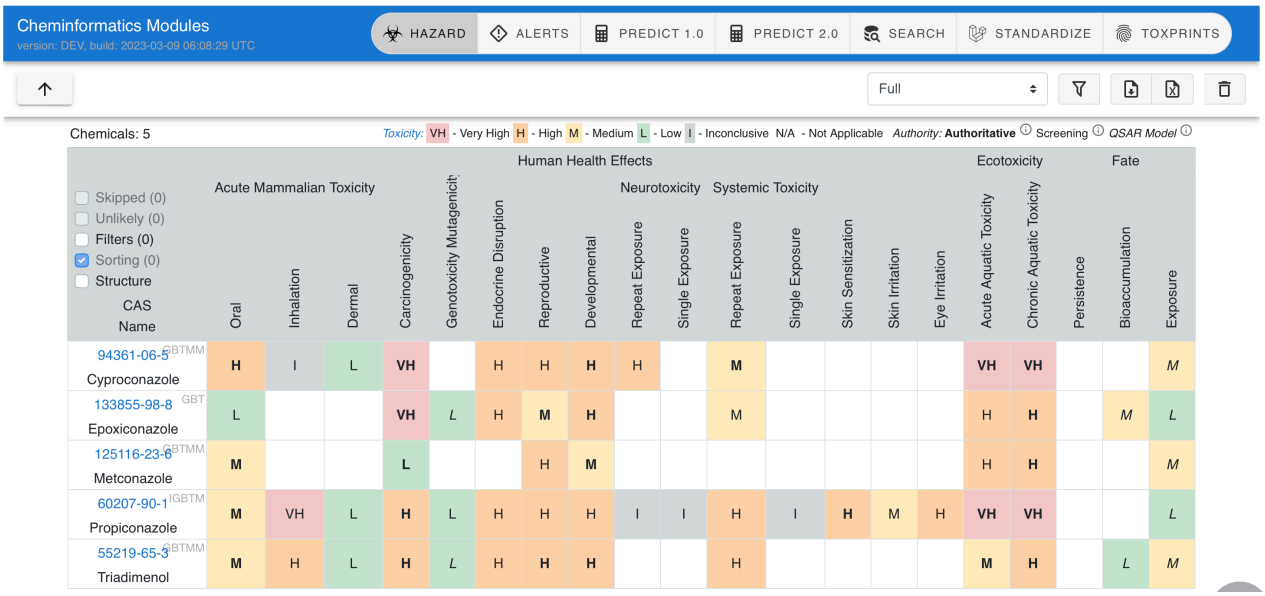
Além disso, a ferramenta de extrapolação *in vitro* para *in vivo* (IVIVE) do ICE traduz concentrações de atividade in vitro para estimativas equivalentes doses in vivo por meio de um processo conhecido como "dosimetria reversa" (Fabian *et al.*, 2019). Utiliza modelos toxicocinéticos fisiologicamente baseados, ou *Physiologically based pharmacokinetic* (PBPK) para extrapolação de medidas experimentais *in vitro* e prever a dose de exposição *in vivo* que resultaria em concentrações internas equivalentes e provocaria respostas biológicas semelhantes em animais ou humanos (Chang *et al.*, 2021). A ferramenta permite que o usuário escolha entre os *endpoints* AC50 ou ACC dos dados cHTS fornecidos pela ferramenta *Curve Surfer*. Em seguida, aplica-se modelagem de dosimetria reversa, utilizando a ferramenta IVIVE, utilizando o pacote R de toxicocinética de alto rendimento (httk) da EPA, para prever uma dose

administrada equivalente, do termo em inglês *equivalent administered dose* (EAD) sob um cenário de via de exposição e dosagem específica para alcançar uma concentração plasmática igual ao *endpoint* selecionado. A interface amigável, que combina os modelos PBTK com dados cHTS, simplifica o processo de modelagem e minimiza a necessidade de conhecimento técnico extenso em programação para realizar as análises (Abedini *et al.*, 2021; Bell *et al.*, 2018; Hines *et al.*, 2022). Desta forma, os níveis de estimados de exposição, baseados na bioatividade *in vitro* e *in silico*, podem ser comparados com exposições humanas reais ou estimadas para se avaliar riscos potenciais à saúde (Breen *et al.*, 2021; Chang *et al.*, 2022).

Diversas ferramentas computacionais e NAMs contribuem com o avanço do estudo de avaliações do risco humano (Benfenati *et al.*, 2019; Brescia *et al.*, 2023; Najjar *et al.*, 2022; Stackelberg and Williams, 2021). Uma delas é a *CompTox Chemicals Dashboard* da EPA (https://comptox.epa.gov/dashboard). Desenvolvida em 2016 para para fornecer acesso público online a dados físico-químicos de substâncias químicas, toxicidade e exposição. Os dados e modelos preditivos dentro da *Dashboard* apoiam a identificação a compostos químicos bioativos que necessitam de testes adicionais, para apoiar uma prioridade da EPA e de acelerar a aquisição de dados de avaliação de toxicidade. Possui informações sobre mais de um milhão de substâncias químicas e continua a expandir em termos do número de substâncias representadas (Williams *et al.*, 2021).

Para melhor interpretar os dados de toxicidade, a EPA também desenvolveu ferramentas de Módulos de Quimioinformática, ou *Cheminformatics Modules* da EPA (https://www.epa.gov/comptox-tools/cheminformatics). Trata-se de um conjunto de ferramentas para analisar dados existentes do perfil de risco de produtos químicos (Vegosen e Martin, 2020), *endpoints* toxicológicos e perfil de compostos químicos utilizando dados HTS de bioatividade *in vitro* do programa ToxCast. Para os fungicidas triazóis, a figura 4 representa uma busca de informações de toxicidade anotadas e classificadas contidas nesta ferramenta computacional.

Figura 4 – Informações de toxicidade de fungicidas triazóis contidas em *Cheminformatics Modules*



Fonte: epa.gov/comptox-tools/cheminformatics.

Nota: Resultados representados com letras em negrito representam dados retirados de fontes de autoridades oficiais como por exemplo EPA, ECHA, IARC, NIOSH, entre outras. Em letras normais representam dados retirados de *screening* fontes (Vegosen e Martin, 2020).

3 OBJETIVOS

O objetivo geral do presente estudo é realizar a avaliação do risco da exposição humana aos fungicidas triazóis por biomonitoramento de dose interna, medições de biomarcadores e integração de dados de *high-throughput screening* (HTS) através de ferramentas de toxicologia computacional utilizando o *Integrated Chemical Environment* (ICE).

3.1 OBJETIVOS ESPECÍFICOS

a) Analisar bioindicador de dose interna por VALLME-CG/MS para detecção e quantificação dos fungicidas triazóis em urina dos voluntários;
b) Analisar biomarcadores de efeitos genotóxicos através do ensaio do citoma de mucosa bucal;
c) Analisar bioindicadores de estresse oxidativo, medindo a peroxidação lipídica e determinando a atividade das enzimas SOD e catalase;
d) Analisar a concentração de ácidos biliares em amostras de plasma para avaliar o efeito hepatotóxico da exposição aos agrotóxicos;
e) Avaliar a função hepática de voluntários masculinos dos grupos rural e urbano por meio da análise das enzimas AST, ALT e γ-GT;
f) Medir os níveis de androstenediona e testosterona total no soro dos voluntários masculinos dos grupos rural e urbano;
g) Analisar os parâmetros bioquímicos colesterol, HDL, LDL, VLDL, triglicerídeos e glicose em amostras de soro;
h) Realizar cálculos do risco utilizando dados de biomonitoramento;
i) Analisar os dados de HTS de ensaios *in vitro* dos fungicidas triazóis e outros ingredientes ativos das formulações dos fungicidas utilizando o a ferramenta *Curve Surfer* do ICE;
j) Comparar os dados de biomonitoramento e predições de exposição dos triazóis e outros ingredientes ativos com EADs determinadas por dosimetria reversa utilizando a ferramenta IVIVE do ICE;

4 MATERIAIS E MÉTODOS

O material e dados obtidos na pesquisa foram utilizados exclusivamente para as finalidades descritas neste estudo. Este trabalho foi submetido e aprovado no Comitê de Ética em Pesquisa da Universidade Federal de Alfenas (UNIFAL-MG), CAAE: 34644620.2.0000.5142, em outubro de 2020 (ANEXO A), para realização das coletas dos dados e as amostras, assim como os resultados do estudo foram publicados sem revelar a identidade dos voluntários.

A fonte dos recursos do financiamento deste projeto é através do Programa de Apoio à Pós-Graduação – PROAP da CAPES para custeio dos materiais, reagentes e padrões analíticos. Para os exames clínicos houve parceria com a Cooperativa Mista Agropecuária de Paraguaçu (COOMAP).

4.1 AMOSTRAGEM

A amostragem foi realizada durante o uso intensivo de fungicidas triazóis, na região sul de Minas Gerais, Brasil, entre dezembro de 2021 e março de 2022. Voluntários com idade acima de 18 anos, que residem e trabalham na zona rural do sul de Minas Gerais e que estejam ambientalmente ou ocupacionalmente expostos aos fungicidas, foram escolhidos como participantes da pesquisa. Os critérios de inclusão abrangeram voluntários do sexo masculino que trabalhavam como cafeicultores, responsáveis pela aplicação dos fungicidas triazóis, e mulheres que residiam na zona rural sem contato direto com a aplicação de agrotóxicos. Esta parte da amostragem foi possível com a colaboração da COOMAP.

Os voluntários do estudo foram divididos em dois grupos: aqueles ocupacionalmente e ambientalmente expostos aos agrotóxicos (n = 140) de áreas rurais, e aqueles não expostos ocupacionalmente (n = 50) de áreas urbanas, servindo como grupo de comparação. Do grupo rural obteve-se n = 88 voluntários do sexo masculino (grupo exposto de homens da zona rural), n = 52 mulheres, das famílias dos voluntários expostos (grupo exposto de mulheres da zona rural). Além disso, foram coletadas amostras de um grupo não exposto ocupacionalmente aos agrotóxicos, residentes da área urbana, n = 25 homens e n = 25 mulheres. Vale ressaltar que o grupo urbano foi selecionado especificamente entre indivíduos saudáveis. Como critério de exclusão, voluntários que apresentavam comorbidades,

como câncer, que pudessem interferir e constituir um possível viés nos resultados, não foram incluídos nos grupos da pesquisa.

Na etapa de coleta de amostras dos voluntários foi aplicado o questionário (APÊNDICE A). O questionário contém perguntas sobre a exposição ocupacional aos agrotóxicos e o estado de saúde dos voluntários. As perguntas foram lidas para os voluntários, e suas respostas foram anotadas. Cada voluntário teve o direito e total liberdade de não responder qualquer pergunta que julgou violar sua privacidade ou causar algum desconforto. Também assinou e ficou com uma cópia do Termo de Consentimento Livre e Esclarecido (APÊNDICE B).

Além do questionário, foram coletadas amostras de sangue, células do epitélio bucal e urina dos voluntários para análises toxicológicas, bioquímicas e hormonais. As amostras de sangue foram coletadas por punção venosa, utilizando tubos sem anticoagulantes (soro) e EDTA (plasma), e processadas imediatamente após a coleta. As amostras de células do epitélio bucal foram obtidas através de um esfregaço do interior das bochechas, utilizando um *swab* de algodão, que foi imerso em solução fisiológica para preservação das células. A coleta de urina foi realizada em um frasco coletor estéril e descartável, com a coleta de uma quantidade aproximada de 50 a 100 mL. Todas as amostras foram transportadas em caixa térmica com gelo artificial reutilizável (Gelox) (TermoGel, São Paulo, Brasil) para o laboratório em temperatura igual ou inferior a 8 °C e armazenadas em um ultra freezer a −70 °C.

Não houve diferença entre a coleta de dados e materiais biológicos entre os grupos da zona rural e da zona urbana, sendo utilizada a mesma metodologia para todos, atendendo os requisitos éticos referentes às pesquisas em seres humanos.

4.2 ANÁLISE DE FUNGICIDAS TRIAZÓIS EM URINA

A coleta das amostras de urina foi realizada para coincidir com a época do ano em que os trabalhadores aplicam os fungicidas triazóis, entre novembro e março. Os triazóis foram detectados e quantificados segundo o método VALLME-CG/MS desenvolvido por Machado *et al*. (2019) e adaptado por Marciano *et al*. (2024). As concentrações encontradas na urina foram analisadas e comparadas estatisticamente após normalização com a creatinina urinária.

4.2.1 Padrões, reagentes e solventes utilizados

Ciproconazol (CIP, pureza 95%) foi obtido da Santa Cruz (EUA). Epoxiconazol (EPX, pureza 99.2%), metconazol (MET, pureza 99.5%), triadimenol (TDN, pureza 98.5%) e Tebuconazol-terc-butil-d9 (TEB-D9) foram obtidos da Sigma-Aldrich® (São Paulo, Brasil). Propiconazol (PRP, mistura de estéreo isômeros, pureza 99%) da linha Pestanal® da Sigma-Aldrich® (São Paulo, Brasil). Enzima β-glucuronidase de *Helix pomatia* (tipo H-2) foi adquirida da Sigma-Aldrich® (Saint Louis, EUA). O metanol grau HPLC utilizado fornecido por Êxodo Científica® (Sumaré, Brasil) e a acetonitrila, também grau HPLC, da Dinâmica® (Indaiatuba, Brasil). O tolueno grau resíduo de praguicidas (pureza 99%) foi adquirido da G rupo Química® (Penha, Brasil). Fosfato de potássio dibásico 98% e ácido ortofosfórico 85% Vetec® (Duque de Caxias, Brasil) e acetato de sódio Synth® (Diadema, Brasil) foram usados. Água ultrapura foi obtida utilizando um sistema de purificação de água Milli-Q Plus (Millipore®, Bedford, EUA).

As soluções estoque (1 g/L) e intermediárias (10 mg/L e 1 mg/L) foram preparadas em metanol. Todas as soluções foram armazenadas a −20 °C.

As curvas analíticas foram preparadas fortificando-se *pool* de urina branco nas seguintes concentrações: 10; 30; 50; 70; 90; 120; 140; 180 e 200 µg/L. As amostras branco foram concedidas por voluntários não expostos ocupacionalmente aos agrotóxicos. Os controles de qualidade baixo, médio e alto (CQB, CQM e CQA, respectivamente) foram as concentrações de 50, 120 e 180 µg/L.

4.2.2 Condições cromatográficas e espectrométricas

Os parâmetros analíticos do método foram estabelecidos com base na metodologia desenvolvida por Machado *et al*. (2019). Os triazóis foram analisados usando um CG-MS (QP 2010 Plus, Shimadzu®, Kyoto, Japão), equipado com um autoamostrador, operando no modo *splitless* a uma temperatura de 300 °C durante a corrida cromatográfica.

Os fungicidas foram separados utilizando uma coluna capilar de 5% fenil e 95% dimetilpolisiloxano *low bleed* (OPTIMA® - 5; 30m × 0,25mm × 0,25m; Macherey-Nagel, Alemanha), com hélio como gás de arraste (99,999%) e um fluxo total de 10 mL/min controlado pelo modo de velocidade linear. O volume de injeção foi de 2 µL. O programa de temperatura aplicado para separar os analitos iniciou a 120 °C,

aumentou para 300 °C a uma taxa de 40 °C/min, e manteve-se a 300 °C por 3 minutos, totalizando uma duração de 7,5 minutos. As condições para o espectrômetro de massas foram configuradas da seguinte maneira: temperatura da interface e da fonte de ionização a 290 °C, operando no modo de ionização por impacto de elétrons a 70 eV, com a análise começando após 3 minutos do início da corrida cromatográfica. As análises foram conduzidas no modo SIM (*Selected Ion Monitoring*), monitorando um fragmento para quantificação e dois para detecção. Foram detectados dois picos nas análises de propiconazol e triadimenol, correspondentes aos seus respectivos isômeros.

4.2.3 Preparo da amostra

Para a hidrólise, transferiu-se 1 mL de urina para um tubo de polipropileno de 15 mL com tampa de rosca. Adicionou-se 100 µL da enzima β-glucuronidase de *Helix pomatia* (diluída na proporção de 1:28 em tampão acetato 0,5 mol/L, pH 5,0). As amostras foram incubadas em uma estufa a 38 °C por 12 horas.

Em seguida, à temperatura ambiente, acrescentou-se 20 µL da solução de padrão interno (TEB-D9) com concentração de 10 mg/L, 2 mL de tampão fosfato (1 mol/L, pH 7,0), 1 mL de acetonitrila e 200 µL de tolueno. A mistura foi agitada vigorosamente por 1 minuto em um vórtex e posteriormente centrifugada a 1650 g por 5 minutos. Transferiram-se 200 µL do sobrenadante para um microtubo de polipropileno de 2 mL para a secagem do solvente em uma centrífuga concentradora/evaporadora a vácuo (Centrivap® Labconco Corporation, Kansas City, EUA) por 20 minutos a 30 °C. No resíduo seco, adicionaram-se 100 µL de tolueno e, após homogeneização, o conteúdo foi transferido para um vial para análise por CG-MS.

4.3 ENSAIO DO CITOMA DE CÉLULAS DE MUCOSA BUCAL

Para o ensaio do citoma, seguiu-se a metodologia para o preparo das lâminas conforme descrito por Silvério *et al*. (2017) e Benedetti *et al*. (2013), e a análise das alterações celulares utilizando o estudo realizado por Thomas *et al*. (2009).

Realizou-se um esfregaço bucal com *swab* de algodão em ambos os lados da bochecha. Após a coleta, o material foi colocado em um tubo de polipropileno de 15

mL contendo 5 mL de solução de cloreto de sódio a 0,9% (m/v) e transportado para o laboratório onde as lâminas foram preparadas.

Para o preparo das lâminas, o *swab* foi descartado e a solução foi centrifugada por 5 minutos a 750 g. Após a centrifugação, 4 mL foram descartados e 5 mL foram reconstituídos novamente com solução de cloreto de sódio (0,9% m/v) e centrifugados nas mesmas condições. Este processo de lavagem das células em suspensão foi repetido três vezes. Finalmente, uma última centrifugação com uma solução fixadora de ácido acético:metanol (1:3, v/v) foi realizada. Após a última etapa com a solução fixadora, aproximadamente 1 mL da suspensão de células foi deixado no tubo e gentilmente movimentado para ressuspender o *pellet* de células. Em seguida, a suspensão foi transferida para duas lâminas, que foram deixadas secar à temperatura ambiente. As lâminas foram coradas com laranja de acridina (Sigma-Aldrich®, São Paulo, Brasil) em uma concentração de 0,001% em água por 5 minutos, enxaguadas em água destilada por 2 minutos e cobertas com uma lamínula.

As análises das lâminas foram realizadas em um microscópio de fluorescência (Leica Microsystems®, Wetzlar, Alemanha) com filtro azul e ampliação de 400x. Para cada voluntário, a frequência dos diferentes tipos de células no ensaio foi representada pelo número de células em um total de 2000 analisadas, sendo contadas 1000 células por lâmina.

Os biomarcadores avaliados foram a frequência de células binucleadas (célula contendo dois núcleos no citoplasma), células com micronúcleos (células com um ou mais fragmentos de núcleo no citoplasma e morte celular), cariorrexe (célula com núcleo desintegrando), cariólise (célula sem núcleo no citoplasma) e picnose (células com núcleo muito reduzido).

4.4 BIOMARCADORES DE ESTRESSE OXIDATIVO

Para avaliar o estresse oxidativo, foram determinadas a peroxidação lipídica e a atividade das enzimas SOD e catalase. Os resultados de cada ensaio foram expressos em relação à concentração de proteínas séricas de cada amostra. Todos os ensaios foram realizados no Laboratório de Bioquímica Clínica e Experimental da UNIFAL-MG.

4.4.1 Peroxidação lipídica

A peroxidação lipídica foi avaliada pela medida de substâncias reativas ao ácido tiobarbitúrico (TBARS) no soro, que reflete indiretamente a produção de malondialdeído (MDA) por fluorimetria (Sinnhuber e Yu, 1977). A concentração de MDA foi estimada usando uma curva padrão com tetraetoxipropano (0,3 µmol a 38,4 µmol) (Sigma-Aldrich®, São Paulo, Brasil). Para cada 150 µL de amostra ou padrão, foram adicionados 750 µL de ácido fosfórico 1,22 M (Vetec®, Duque de Caxias, Brasil), 1350 µL de água e 750 µL de TBARS 0,67% em ácido acético 50% (Dinâmica® Química Contemporânea, Indaiatuba, Brasil). Em seguida, a mistura foi homogeneizada por 30 segundos e as amostras foram incubadas em banho-maria a 95 °C por 60 minutos. Após o banho de água fervente, os tubos com as amostras foram resfriados em banho de gelo. Após o resfriamento, 1800 µL de metanol e 1000 µL de NaOH 1 M foram adicionados para cada alíquota de 200 µL de amostra ou padrão. As leituras foram realizadas em um espectrofluorímetro (Cary Eclipse Fluorimeter, Varian, Walnut Creek, EUA) em comprimentos de onda de excitação e emissão de 532 nm e 553 nm, respectivamente.

4.4.2 Superóxido dismutase (SOD)

A atividade da SOD foi medida em amostras de soro usando ensaio colorimétrico (Ōyanagui, 1984). Alíquotas de soro foram incubadas com 0,01 M de hidroxilamina, 1 mM de hipoxantina e 0,0515 U/ml de xantina oxidase da Sigma-Aldrich® (São Paulo, Brasil) a 37 °C por 30 minutos no escuro. Após a incubação, adicionou-se 1000 µL de uma solução contendo ácido sulfanílico, α-naftilendiamina e ácido acético glacial (Dinâmica® Química Contemporânea, Indaiatuba, Brasil) e a mistura foi deixada à temperatura ambiente por 20 minutos. O espectrofotômetro foi ajustado para 550 nm (Espectrofotômetro UV-Vis com Controle de Temperatura Peltier, Madison, Thermo Scientific, EUA). A atividade enzimática foi calculada com base no princípio de que 1 unidade da enzima produz 50% de inibição na reação.

4.4.3 Catalase

A atividade da catalase foi medida em amostras de soro utilizando um espectrofotômetro com temperatura controlada a 37 °C, monitorando o consumo de H_2O_2 a cada minuto em 240 nm (Aebi, 1984). Alíquotas da amostra foram incubadas em tampão PBS com pH 7,4. A reação foi iniciada com a adição de 10 mM de H_2O_2, e a absorbância foi registrada continuamente por um minuto dentro do espectrofotômetro (Espectrofotômetro UV-Vis com Controle de Temperatura Peltier, Madison, Thermo Scientific, EUA). A cinética de decomposição do H_2O_2 foi determinada usando seu coeficiente de extinção molar em 240 nm (43.6 M^{-1} cm^{-1}). Os resultados foram expressos em U/mg de proteína, onde U corresponde à atividade da enzima que promove a hidrólise de 1 µmol de H_2O_2 por minuto.

4.4.4 Proteínas totais séricas

A análise das proteínas totais foi realizada utilizando o método de Bradford (Zaia, Zaia e Lichtig, 1998). Esse método emprega o corante azul de *Coomassie Brilliant* para medir as proteínas séricas totais. Uma solução padrão de proteína de albumina sérica bovina (BSA) da Sigma-Aldrich® (São Paulo, Brasil) foi utilizada para gerar uma curva analítica, de 0,2 a 1,0 mg/mL, para a quantificação das amostras. A absorbância foi medida a 595 nm usando um espectrofotômetro (Genesys 10S Series, Madison, Thermo Scientific, EUA).

4.5 ANÁLISE DE ÁCIDOS BILIARES

A análise dos ácidos biliares foi realizada baseando-se no método desenvolvido por Wang *et al*. (2015), com modificações feitas por Machado *et al*. (2021) e Silveira *et al*. (2022).

4.5.1 Padrões, reagentes e solventes utilizados

Ácido cólico (CA, pureza $\geqslant$ 98%), ácido deoxicólico (DCA, pureza $\geqslant$ 98%), ácido glicodesoxicólico (GDCA, pureza $\geqslant$ 97%), ácido taurocólico (TCA, pureza $\geqslant$

97%) e ácido taurodeoxicólico (TDCA, pureza ⩾ 97%) foram adquiridos da Sigma-Aldrich® (Steinheim, Alemanha). O metanol grau HPLC utilizado foi o Êxodo Científica (Sumaré, Brasil), acetonitrila e álcool etílico grau HPLC, da Dinâmica® (Química Contemporânea, Brasil). Acetato de amônio 98% e ácido fórmico 85% (Vetec®, Duque de Caxias, Brasil) foram usados. Água ultrapura foi obtida utilizando um sistema de purificação de água Milli-Q Plus (Millipore®, Bedford, EUA).

As soluções estoque (1 g/L) e intermediárias (10 mg/L e 1 mg/L) foram preparadas em metanol. Todas as soluções foram armazenadas a −20 °C.

As curvas analíticas foram preparadas fortificando plasma sanguíneo nas seguintes concentrações: 10, 25, 50, 100, 250, 500 µg/L. Foi utilizado plasma não fortificado para amostras branco.

4.5.2 Condições cromatográficas e espectrométricas

As amostras foram analisadas utilizando um UHPLC-MS/MS (LCMS-8030, Shimadzu®, Kyoto, Japão), acoplado a um analisador de massas do tipo triplo quadrupolo, com interface ESI, operando no modo negativo (ESI, −3.5 kV). A separação cromatográfica foi realizada utilizando a coluna cromatográfica NST 18100 (150 mm x 4,6 mm; 5 µm), precedida por uma pré-coluna Supelcoguard LC-18 (10 mm x 4,6 mm; 5 µm), com o forno da coluna, bloco de aquecimento e linha de dessolvatação em temperaturas ajustadas para 35, 400 e 250 °C, respectivamente. As taxas de fluxo de gás de nebulização e secagem (N_2) foram de 2 e 15 L/min, respectivamente. O volume de injeção foi de 10 µL. O tempo total da corrida foi de 17,5 minutos.

A fase móvel consistiu de metanol/acetato de amônio 5 mmol/L, com 0,012% de ácido fórmico (solução A) e água ultrapura/acetato de amônio 5 mmol/L com 0,012% de ácido fórmico (solução B) na proporção de 90:10 (v/v), com uma vazão total de 0,3 mL/min.

4.5.3 Preparo da amostra

Em um microtubo de polipropileno de 2 mL, adicionou-se 500 µL de uma solução de álcool etílico 75% a 200 µL de plasma. O microtubo foi agitado em vórtex por 1 minuto e, em seguida, centrifugado a 18.928 g por 10 minutos. Posteriormente,

transferiu-se 350 µL do sobrenadante para outro microtubo, e adicionou-se 1 mL de acetonitrila gelada. O microtubo foi novamente agitado em vórtex e centrifugado nas mesmas condições. Após a centrifugação, transferiram-se 600 µL do sobrenadante para outro microtubo e submetidos à secagem por 40 minutos a 80 °C em uma centrífuga concentradora/evaporadora a vácuo (Centrivap® Labconco Corporation, Kansas City, EUA). O resíduo foi reconstituído com 100 µL de fase móvel na proporção de 90:10 (v/v) da solucao A e da solucao B, homogeneizado e transferido para um vial para análise por UHPLC-MS/MS.

4.6 ENZIMAS HEPÁTICAS

A avaliação das enzimas hepáticas foi realizada com voluntários masculinos da área rural (n = 88), e voluntários masculinos da área urbana (n = 25). As análises foram conduzidas no Laboratório Central de Análises Clínicas da UNIFAL-MG, assegurando resultados padronizados. A medição das atividades enzimáticas utilizou métodos cinéticos UV automatizados para AST (Bergmeyer *et al*., 1986a), ALT (Bergmeyer *et al*., 1986b) e γ-GT (Siekmann *et al.*, 2002)

As faixas de referência para as enzimas são: AST de 5 a 34 U/L, ALT de 0 a 55 U/L e γ-GT para homens.

4.7 PARÂMETROS BIOQUÍMICOS E NÍVEIS DE ANDROSTENEDIONA E TESTOSTERONA

A avaliação dos parâmetros bioquímicos foi realizada com voluntários masculinos (n = 88) e femininos (n = 52) de áreas rurais, juntamente com voluntários masculinos de áreas urbanas (n = 25). Essas análises foram realizadas no Laboratório Central de Análises Clínicas da UNIFAL-MG.

Os níveis de colesterol, lipoproteína de alta densidade (HDL), lipoproteína de baixa densidade (LDL), lipoproteína de muito baixa densidade (VLDL) (fórmula de Friedewald), triglicerídeos e glicose foram medidos no soro usando métodos enzimáticos automatizados (Trinder).

As análises hormonais para testosterona total e androstenediona foram conduzidas em amostras de soro obtidas de voluntários masculinos das áreas rurais (n = 88) e urbanas (n = 25). Esta análise teve como objetivo investigar a alteração dos

hormônios masculinos após a exposição aos triazóis e o impacto dessa exposição ocupacional. As medições foram realizadas no mesmo laboratório utilizando dois ensaios: o ensaio automatizado Access Testosterone (Beckman Coulter Inc., Fullerton, CA, EUA), um ensaio imunométrico de ligação competitiva para testosterona (Dittadi *et al.*, 2018), e o ensaio Immulite 2000 (Siemens Medical Solutions Inc., Malvern, PA, EUA), um ensaio imunoenzimático quimioluminescente (Owen e Roberts, 2007).

Os valores de referência para androstenediona em adultos masculinos entre 18 e 66 anos variam de 0,6 a 3,1 ng/dL. Os valores de referência para testosterona total são de 175,0 a 781,0 ng/dL.

4.8 CÁLCULOS DE RISCO UTILIZANDO DADOS DE BIOMONITORAMENTO

Para interpretar os níveis urinários do bioindicador de dose interna no contexto da avaliação do risco, foi calculada a *Estimated Daily Intake* (EDI) de triazóis de acordo com a Eq. (1), que foi semelhante àquelas utilizadas em estudos recentes (Ferreira *et al.*, 2021; Li *et al.*, 2022; Šulc *et al.*, 2022).

$$\mathrm{EDI} = \frac{C \,.\, CE}{pc \,.\, F} \tag{1}$$

EDI: *Estimated Daily Intake* (µg/kg-pc/dia); C: concentração de triazol na urina (µg/g creatinina); CE: valor de referência para excreção de creatinina em urina derivada de adultos no Brasil (1,22 g creatinina/dia) (Mill *et al.*, 2012); pc: peso corporal médio de homens e mulheres do grupo exposto (kg); F: fator de excreção urinária de triazol (0,17 para epoxiconazol (EFSA, 2008a) (EFSA, 2008a), 0,27 para ciproconazol (EFSA, 2010) e 0,5 para triadimenol (EFSA, 2008b)).

A EDI foi dividida pela Ingestão Diária Aceitável (IDA) para calcular o *Hazard Quotient* (HQ), que representa a relação entre a exposição estimada a uma substância, representado pela EDI e os níveis nos quais nenhum efeito tóxico é esperado, definido pela IDA (Katsikantami *et al.*, 2019). O HQ permite avaliar o risco de exposição aos agrotóxicos. Se o HQ for menor que um, é considerado de baixo risco à saúde (Fernández, Pardo, Adam-Cervera, *et al.*, 2020).

$$\mathrm{HQ} = \frac{EDI}{\mathrm{IDA}} \quad (2)$$

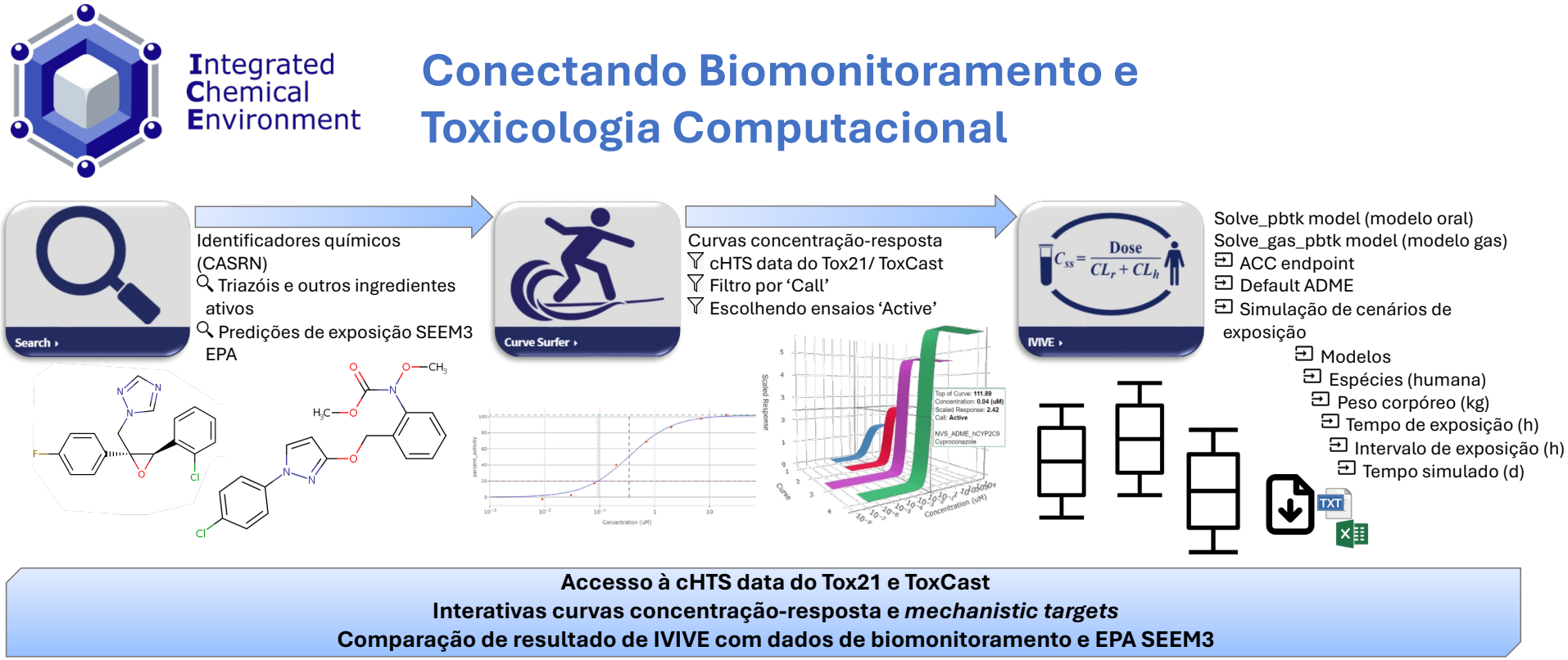
4.9 TOXICOLOGIA COMPUTACIONAL UTILIZANDO O *INTEGRATED CHEMICAL ENVIRONMENT* (ICE)

O fluxo de trabalho, ou do termo em ingles *workflow*, utilizado no ICE (https://ice.ntp.niehs.nih.gov/) versão 4.0.2 é ilustrado na Figura 5.

Inicialmente, a ferramenta *Search* foi utilizada para inserir os identificadores químicos (CASRNs) dos triazóis detectados. Além disso, também foram incluídos outros princípios ativos presentes no produtos de agrotóxicos relatados como utilizados pelos agricultores no questionário (azoxistrobina, piraclostrobina e tiametoxam, associados aos produtos OPERA®, PrioriXtra® e Verdadero® respectivamente). As predições de exposição *Systematic Empirical Evaluation of Models* (SEEM3) da EPA foram acessadas para os triazóis detectados e os outros ingredientes ativos, e os resultados da busca foram exportados para um arquivo Excel que contém o percentil 5 (P5), mediana e percentil 95 (P95) (Ring *et al.*, 2019)

Os compostos químicos foram então enviados para a ferramenta *Curve Surfer* e todos os conjuntos de dados cHTS foram selecionados. Na página de resultados, o filtro aplicado foi por '*Call*', escolhendo a opção '*Active*'. Todos os ensaios ativos selecionados para os agrotóxicos foram enviados para a ferramenta IVIVE. Na página de entrada da ferramenta IVIVE, foram testados o solve_pbtk para exposições orais e o solve_gas_pbtk para exposições por inalação. Para todas as análises, o *endpoint* escolhido foi o ACC, e a fonte do parâmetro ADME (absorção química, distribuição, metabolismo e excreção) foi definida como padrão (ou seja, experimental, se disponível, caso contrário, prevista computacionalmente).

Figura 5 – ICE *workflow* aplicado no estudo



Fonte: Do Autor.

Nota: Integrated Chemical Environment (ICE) versão 4.0.2. cHTS: *curated high-throughput screening*. ACC: *activity concentration at the activity threshold cutoff*. ADME: *chemical absorption, distribution, metabolism, and excretion*. EADs: *equivalent administered doses*. SEEM3: *Systematic Empirical Evaluation of Models*.

Para avaliar a exposição oral, foi considerada uma dose de intervalo de 10 horas para simular o padrão de consumo de indivíduos que comem produtos agrícolas cultivados localmente duas vezes ao dia. Este padrão de consumo é particularmente relevante para aqueles que residem perto de plantações de café e outras culturas, onde existe potencial à dispersão de sprays de agrotóxicos. Neste contexto, foi selecionado um período de 150 dias, de novembro a março, para corresponder à época de aplicação de fungicidas na região. Esses parâmetros são projetados para simular a probabilidade de comunidades rurais serem expostas diariamente aos agrotóxicos por via oral. Quanto ao peso corporal escolhido, objetivou-se representar o peso corporal médio dos homens (75,8 kg) e das mulheres (73,1 kg) do grupo rural como média entre os dois (74,45 kg). Como não avaliou-se o biomonitoramento para os outros três princípios ativos, o mesmo valor médio foi utilizado para avaliar um cenário de exposição mais amplo.

Para comparar os resultados dos EADs da modelagem de dosimetria reversa na ferramenta IVIVE com os dados humanos do estudo, presume-se que toda a EDI se origina da via oral ou inalatória. Esta suposição é necessária porque o biomarcador urinário utilizado para calcular a EDI não consegue diferenciar as vias de exposição. Os dados de biomonitoramento incluem todas as vias de exposição: oral, inalatória e dérmica, tornando esta suposição essencial para a aplicação dos modelos de exposição oral e inalatória. Para exposição oral, a EDI foram convertidas para mg/kg/dose.

Por outro lado, para o modelo de gás, os dados de biomonitoramento da EDI foram convertidos em concentrações atmosféricas (C ar), uma vez que os EADs gerados pela ferramenta IVIVE são definidos em µM/dose. A Eq. (3) (López *et al.*, 2021) foi aplicada para estimar uma dose diária de inalação a partir das concentrações medidas no ar. Este procedimento é vital para a utilização dos dados de triazóis urinários, que indicam a exposição total a estes fungicidas, mas sem a disponibilidade de medições diretas de agrotóxicos no ar. Portanto, a Eq. (3) simplesmente isola a concentração do ar (C ar), permitindo que os dados de biomonitoramento atuem como parâmetro de entrada para executar o modelo de gás no ICE e possibilitando a comparação dos EADs da ferramenta IVIVE com os dados humanos disponíveis no estudo.

$$\text{C ar} = \frac{Dose\ inalatória\ diária\ .\ pc}{\text{IR . ET}} \quad (3)$$

A dose inalatória diária é considerada igual a EDI (µg/kg-pc/dose) para os voluntários do sexo masculino do grupo rural. A concentração estimada no ar (C ar estimado) é em µg/m^3, com IR indicando a taxa de inalação de 0,053 m³/h/pc (EFSA *et al.*, 2022), pc representando o peso corporal médio dos trabalhadores agrícolas (75,8 kg), e ET sendo o tempo exposição (7 horas). Utilizando a concentração de ar estimada em µg/m^3, a conversão para unidades µM é realizada através da aplicação do peso molecular dos triazóis detectados.

Para avaliar a exposição por inalação na ferramenta IVIVE, o intervalo de exposição foi definido para 24 horas e a duração da exposição foi de 7 horas (tempo médio de aplicação de agrotóxicos por dia pelos agricultores). A duração da simulação simulada foi de 4 dias (número médio de dias aplicando ativamente fungicidas).

4.10 ANÁLISES ESTATÍSTICAS

As análises estatísticas foram realizadas no Software Estatístico R, versão 4.3.2. Para a análise dos triazóis urinários por CG-MS, utilizou-se o teste de Grubbs para identificar e remover dados *outliers*, e as pressuposições básicas do modelo de regressão linear, como normalidade, independência e homogeneidade de variâncias, foram verificadas pelos testes de Shapiro-Wilk, Box-Pierce e Breusch-Pagan, respectivamente.

Na análise dos biomarcadores, o teste de Shapiro-Wilk avaliou a normalidade dos dados. Devido à distribuição não normal dos dados, foram utilizados testes não paramétricos. Para comparações múltiplas, empregou-se o teste de Kruskal-Wallis, seguido pelo teste de Dunn para análise post hoc. O teste de Mann-Whitney foi utilizado para comparações pareadas entre dois grupos. Além disso, a correlação de Spearman foi aplicada para avaliar a relação entre os níveis hormonais e os parâmetros bioquímicos em voluntários do sexo masculino. Um nível de significância de 5% foi estabelecido para todas as análises.

5 RESULTADOS E DISCUSSÕES

5.1 CARACTERÍSTICAS DA POPULAÇÃO DO ESTUDO

A população do estudo consistiu de 190 participantes que foram divididos em dois grupos de estudo por sua exposição aos agrotóxicos: residentes rurais expostos aos agrotóxicos (n = 140) e adultos urbanos saudáveis (n = 50) como grupo de comparação. Suas características estão descritas na Tabela 1.

Tabela 1 – Características do grupo rural e urbano do Sul de Minas Gerais

Características	Zona rural (n = 140)	Zona urbana (n = 50)
Sexo n (%)		
Masculino	88 (63)	25 (50)
Feminino	52 (47)	25 (50)
Idade (média ± dp)	46,0 ± 12.6	25,5 ± 7.1
Peso corpóreo (média ± dp)		
Masculino	75,8 ± 11,7	75,3 ± 12,8
Feminino	73,1± 15,0	66,8 ± 10,5
Consumo de álcool n (%)	38 (27)	31 (62)
Tabagismo n (%)	21 (15)	8 (16)
Nível educacional n (%)		
Fundamental incompleto	85 (60,7)	-
Apenas fundamental completo	8 (5,7)	-
Ensino médio completo	40 (28,6)	44 (88)
Ensino superior	7 (5,0)	6 (12)
Complicações de saúde relatadas n (%)		
Cardiovascular	42 (30)	4 (8)
Sistema nervoso	71 (51)	26 (52)
Digestivo	59 (42)	17 (34)
Respiratório	42 (30)	14 (28)
Auditivo	30 (21)	4 (8)
Pele e mucosa	28 (20)	0
Urinário	18 (13)	3 (6)
Covid-19	8 (6)	20 (40)

Fonte: Do Autor.

Todos os voluntários masculinos do grupo exposto (n = 88) trabalham diretamente na agricultura e relataram a aplicação de agrotóxicos, principalmente fungicidas triazóis, uma vez que o cultivo de café predomina na região (Volsi *et al.*, 2019). As mulheres residentes nas áreas rurais (n = 52) também estão expostas ambientalmente aos agrotóxicos. Elas estão sujeitas à exposição através do consumo de alimentos cultivados localmente que podem conter resíduos de agrotóxicos, durante a lavagem de roupas contaminadas dos maridos e pela proximidade à deriva de agrotóxicos.

A média e desvio padrão de idade dos residentes rurais foi de 46,0 ± 12,6 anos, e 25,5 ± 7,1 anos para residentes urbanos. Apesar da idade do grupo controle ser mais jovem, todos os participantes deste grupo atenderam aos critérios de inclusão para este grupo de estudo, estando em bom estado de saúde e não estando ocupacionalmente expostos aos agrotóxicos. Em estudos anteriores na própria região de estudo, o grupo da zona urbana com essas características mostrou-se um grupo de controle confiável para comparações (Machado *et al.*, 2021; Silvério *et al.*, 2017).

Comparando as variáveis estudadas, o consumo de álcool foi menor no grupo exposto (27%) do que no grupo controle (62%). E a proporção de fumantes foi praticamente a mesma entre os grupos, sendo o grupo exposto (15%) e o grupo não exposto (16%). Entretanto, grandes diferenças são observadas em relação à escolaridade. O grupo da zona rural apresenta alto índice de baixa escolaridade, com 60,7% dos voluntários sem sequer ter concluído o ensino fundamental. E 5,7% relataram ter apenas o ensino fundamental completo. Enquanto isso, no grupo da área urbana, todos os voluntários relataram ter concluído o ensino médio (88%) com uma taxa muito maior do que na área rural (28,6%).

Em relação aos sintomas clínicos autorreferidos durante as entrevistas na aplicação do questionário, foram observadas diferenças significativas nos sintomas clínicos autorreferidos entre os grupos, especialmente em termos de alterações no sistema cardiovascular, bem como na pele e nas mucosas. No grupo exposto, 30% dos participantes relataram alterações no sistema cardiovascular, em comparação com apenas 8% no grupo urbano. Além disso, 20% dos participantes do grupo exposto relataram alterações na pele e nas mucosas, enquanto nenhum relato similar foi feito pelo grupo urbano. Esses resultados podem ser atribuídos aos efeitos irritantes que os agrotóxicos exercem sobre a pele e os tecidos dérmicos (Damalas e Koutroubas, 2016). Contudo, esses efeitos tóxicos não são exclusivos dos fungicidas triazóis, mas

também ocorrem com exposições a outras classes de agrotóxicos (Silva *et al.*, 2023; Thundiyil *et al.*, 2008).

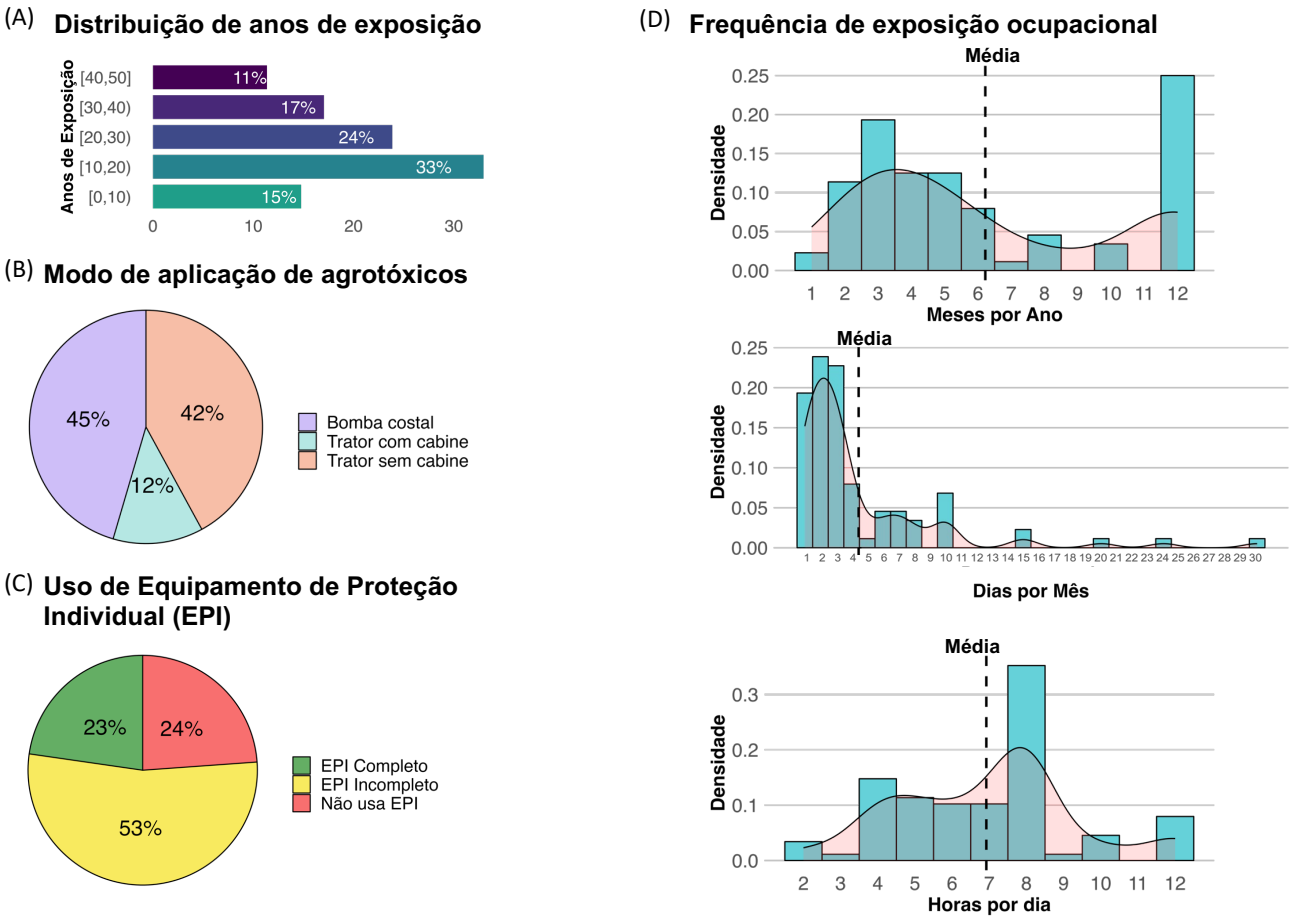
5.2 AVALIAÇÃO DA EXPOSIÇÃO AOS FUNGICIDAS TRIAZÓIS

5.2.1 Avaliação das condições de exposição aos fungicidas triazóis

Os dados de exposição ocupacional coletados por meio do questionário do grupo de agricultores (n = 88) estão resumidos na Figura 6.

Os fungicidas triazóis mais relatados usados pelos trabalhadores da região foram principalmente três produtos comerciais: OPERA® (66%), PrioriXtra® (28%) e Verdadero® (5%), que contêm os seguintes ingredientes ativos: epoxiconazol e piraclostrobina; ciproconazol e azoxistrobina; e ciproconazol tiametoxam, respectivamente.

Figura 6 – Condições de exposição dos agricultores (n = 88)



Fonte: Do Autor.

Nota: (A) Distribuição dos anos de exposição aos agrotóxicos. (B) Modo de aplicação de agrotóxicos autorrelatadas pelos agricultores. (C) Uso de equipamentos de proteção individual (EPI) autorrelatados pelos agricultores. (D) Distribuição da exposição ocupacional aos agrotóxicos em meses por ano (média = 6,2 meses), dias por mês (média = 4,4 dias) e horas por dia (média = 6,9 horas).

Em relação à exposição ocupacional, os dados indicam que 33% dos agricultores tiveram entre 10 e 20 anos de exposição, representando um perfil de exposição crônica entre os agricultores. Notavelmente, 11% dos agricultores foram expostos durante o período mais longo, de 40 a 50 anos, e apenas 15% durante o período mais curto, de 0 a 10 anos. Em termos de métodos de aplicação de agrotóxicos, 45% dos trabalhadores relataram usar bomba costal, 42% usaram trator sem cabine de proteção e 13% usaram trator com cabine. O uso de bomba costal para aplicação de fungicidas é uma preocupação significativa devido à prática generalizada entre os agricultores, o que aumenta significativamente o risco de exposição por inalação. É importante ressaltar que o rótulo do produto OPERA®, um dos produtos mais utilizados, afirma explicitamente que este método de aplicação não é permitido em plantações de café (BASF, 2023).

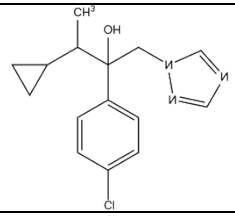
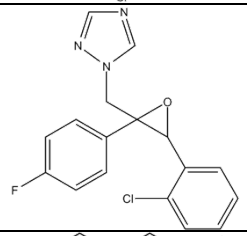
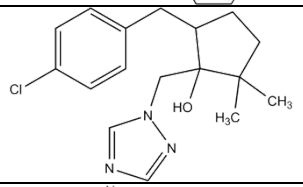
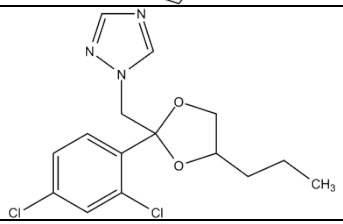
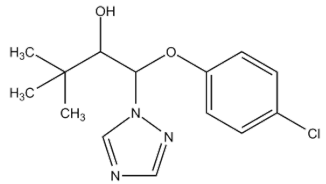
O uso total de equipamentos de proteção individual (EPI), que incluem roupas impermeáveis a agrotóxicos, botas impermeáveis, luvas impermeáveis, máscara e viseira protetora, foi relatado por apenas 23% dos agricultores, enquanto 53% relataram usar pelo menos algum equipamento e 24 % relataram não usar nenhum equipamento. No entanto, neste estudo, não foi observada diferença nos biomarcadores de efeitos medidos neste estudo entre o uso completo de EPI e o uso incompleto ou não uso, sugerindo o potencial de notificação incorreta no questionário pelos voluntários.

As condições de exposição ocupacional relatadas neste estudo são consistentes com os resultados de pesquisas anteriores sobre exposição aos agrotóxicos na região sul de Minas Gerais (Machado *et al.*, 2021; Silvério *et al.*, 2017). A relutância entre os agricultores em usar consistentemente EPI decorre de várias questões complexas, incluindo o desconforto associado ao uso, limitações financeiras que afetam a sua compra e uma falta geral de educação e formação, o que dificulta a compreensão e a adesão aos protocolos de segurança (Abreu e Alonzo, 2014; Santana *et al.*, 2016). Estes elementos sublinham a necessidade de esforços contínuos para educar e equipar os agricultores com os meios para se protegerem, enfatizando o papel da comunicação de riscos, gestão de riscos e implementação de medidas para reduzir os riscos de exposição ocupacional aos agrotóxicos.

5.2.2 Validação do método de triazóis urinários por CG-MS

Na Tabela 2 estão apresentados dados utilizados para análise dos triazóis por CG-MS, entre eles os íons monitorados em razão da sua massa/carga (m/z).

Tabela 2 – Fungicidas triazóis e íons monitorados no CG-MS

Triazol	Fórmula molecular	Massa molecular (g/mol)	Íons monitorados	Tempo de retenção (minutos)	Estrutura química
Ciproconazol	$C_{15}H_{18}ClN_3O$	291,78	222*, 139, 224	4,65	
Epoxiconazol	$C_{17}H_{13}ClFN_3O$	329,76	192*, 165, 138	5,15	
Metconazol	$C_{17}H_{22}ClN_3O$	319,83	125*, 70, 83	5,70	
Propiconazol	$C_{15}H_{17}Cl_2N_3O_2$	342,22	173*, 259, 69	5,16	
Triadimenol	$C_{14}H_{18}ClN_3O_2$	295,76	112*, 128, 168	4,266	

Fonte: Do Autor.
Nota: *Fragmento de quantificação e os outros dois para detecção.

Com base no estudo anterior de Machado *et al*. (2019) a faixa linear do método deste estudo foi estabelecida entre 10 e 200 µg/L. Inicialmente avaliou-se a presença de observações discrepantes (*outliers*) em todos os valores de respostas (área de pico) conforme as concentrações utilizadas como calibradores da curva analítica (10, 30, 50, 70, 90, 120, 140, 180 e 200 µg/L) com aplicação do teste de Grubbs ao nível de 5% de significância, sendo as observações consideradas outliers retiradas dos dados.

Para avaliar os parâmetros de validação parcial foram utilizados guias nacionais e internacionais (BRASIL, 2012, 2017; EMA, 2022; FDA, 2018).

Analisou-se a linearidade da curva analítica de regressão linear simples através do coeficiente de determinação (R^2) e a não violação das pressuposições básicas da regressão: normalidade, independência e homocedasticidade de variância (EMA, 2022; Machado *et al*., 2019). Para verificação de normalidade foi aplicado o teste de Shapiro-Wilk, para independência os testes de Box-Pearce e para análise da homocedasticidade o teste de Breusch-Pagan e o coeficiente de determinação são apresentados na Tabela 3.

Tabela 3 – Avaliação da linearidade do método pela análise das pressuposições básicas da regressão e coeficiente de determinação (R^2) da curva analítica

Triazol	Shapiro- Wilk	Box-Pearce	Breusch- Pagan	R^2
Ciproconazol	0,1408	0,0655	0,1057	0,9905
Epoxiconazol	0,7469	0,3288	0,0800	0,9884
Metconazol	0,8943	0,1881	0,2799	0,9911
Propiconazol	0,2134	0,5973	0,3377	0,9872
Triadimenol	0,0726	0,1777	0,2511	0,9927

Fonte: Do Autor.
Nota: Shapiro-Wilk (normalidade), Box-Pearce (independência) e Breusch-Pagan (homocedasticidade). Valor-p > 0,05 = Resultados não significativos. Com significância de 5%.

Portanto, observou-se que nenhuma pressuposição da regressão foi violada para as curvas ajustadas, de modo que as conclusões obtidas a partir das mesmas são válidas. O R^2 demonstra que os dados estão satisfatoriamente ajustados ao modelo linear.

Assim, foi ajustado o modelo de regressão linear simples para todos os analitos, sem realizar a conversão dos dados das respostas do sinal (área de pico) para

logaritmo das médias das respostas ou inverso da média das respostas como foi executado no trabalho de Machado *et al*. (2019). Isto pode ser explicado por utilizar uma menor faixa linear, tendo o limite superior de quantificação definido em 200 µg/L ao invés de 650 µg/L, devido as concentrações mais elevadas tenderem a influenciar mais o modelo de regressão linear (heterogeneidade) do que os pequenos desvios associados às concentrações mais baixas, sendo (Karnes, Shiu e Shah, 1991).

O limite inferior de quantificação (LIQ) foi considerado a menor concentração da curva analítica que apresentou precisão e exatidão. O limite de detecção (LD) foi determinado de forma empírica, em que concentrações decrescentes inferiores ao LIQ foram testadas até o menor valor que foi possível distinguir sinal (área do pico) comparado com a amostra branco (BRASIL, 2017; EMA, 2022). Os parâmetros da curva analítica, a faixa linear e o LD estão apresentados na Tabela 4.

Tabela 4 – Estimativa dos parâmetros da curva analítica do método de triazóis urinários por CG-MS

Triazol	Parâmetros*	Estimativas	p-valor**	Faixa linear (µg/L)	Limite de detecção (µg/L)
Ciproconazol	a	0,0230	< 0,001	10 - 200	2
	b	0,4178	< 0,001		
Epoxiconazol	a	0,0078	< 0,001	30 - 200	5
	b	-0,0782	< 0,001		
Metconazol	a	0,0198	< 0,001	10 - 200	2
	b	0,1660	< 0,001		
Propiconazol	a	0,0519	< 0,001	30 - 200	5
	b	3,4914	< 0,001		
Triadimenol	a	0,0208	< 0,001	10 - 200	2
	b	0,1474	< 0,001		

Fonte: Do Autor.
Nota: *Parâmetros da equação de regressão linear: $y=ax+b$, em que *a* representa a inclinação da reta e *b* o intercepto no eixo das ordenadas; **Teste t, com significância de 5%.

A exatidão e a precisão do método foram verificadas através dos experimentos de repetibilidade (intra-corridas e inter-corridas), e, em cada corrida cinco réplicas foram analisadas, nas concentrações referentes aos pontos LIQ de cada analito, CQB, CQM e CQA.

A precisão foi expressa como desvio padrão relativo (DPR%) Eq. (4), com valores não excedendo variação de 15%, exceto para o LIQ, para o qual foram admitidos valores iguais ou inferiores a 20% (BRASIL, 2012; FDA, 2018).

$$\mathrm{DPR}(\%) = \frac{\text{desvio padrão}}{\text{concentração experimental}} \text{ x } 100 \quad (4)$$

A Tabela 5 mostram os intervalos de DPR% obtidos, usando matriz branco fortificadas com os analitos, intra-corridas e inter-corridas.

Tabela 5 – Desvios padrões relativos (%) do método de triazóis em urinários por CG-MS

	Intra-corridas				Inter-corridas			
Triazol	LIQ	CQB	CQM	CQA	LIQ	CQB	CQM	CQA
Ciproconazol	1,28	2,84	9,96	8,13	2,12	1,31	7,10	8,19
Epoxiconazol	9,00	5,62	6,81	10,37	13,12	7,81	9,10	10,11
Metconazol	14,57	7,62	11,53	5,20	13,87	9,15	12,04	4,08
Propiconazol	3,58	3,16	5,99	6,46	5,11	5,10	8,15	9,11
Triadimenol	13,64	7,84	13,52	4,67	12,51	5,32	8,10	7,13

Fonte: Do Autor.
Nota: CQB: 50 µg/L; CQM: 120 µg/L; CQA: 180 µg/L.

A exatidão do método foi expressa como erro padrão relativo (EPR%) Eq. (5), com valores não excedendo variação de ±15%, exceto para o LIQ, para o qual foram admitidos valores até ±20% para ambos os parâmetros.

$$\mathrm{EPR}(\%) = \frac{(\text{concentração média} - \text{valor nominal})}{\text{valor nominal}} \text{ x } 100 \quad (5)$$

A Tabela 6 mostram os intervalos de EPR% obtidos, usando matriz branco fortificadas com os analitos, intra-corridas e inter-corridas no LIQ, CQB, CQM e CQA (BRASIL, 2012; FDA, 2018).

Tabela 6 – Erros padrões relativos (%) do método de triazóis em urinários por CG-MS

	Intra-corridas				Inter-corridas			
Triazol	LIQ	CQB	CQM	CQA	LIQ	CQB	CQM	CQA
Ciproconazol	2,46	8,35	-4,42	2,68	-5,10	7,15	-2,25	1,14
Epoxiconazol	20,00	-9,66	-10,34	3,65	18,25	13,78	11,55	9,25
Metconazol	14,94	10,09	-6,37	5,89	9,13	8,85	-7,55	6,35
Propiconazol	-15,34	15,85	0,57	5,56	-14,25	12,28	3,45	11,45
Triadimenol	-6,48	9,29	-6,72	1,79	-7,31	8,21	-5,12	1,12

Fonte: Do Autor.
Nota: CQB: 50 µg/L; CQM: 120 µg/L; CQA: 180 µg/L.

Nos testes de estabilidade dos analitos na amostra biológica testou-se o armazenamento em freezer (−26 °C) com diferentes intervalos de tempo.

Os CQB, CQM e CQA (BRASIL, 2012) foram fortificados e aliquotados em tubos de polipropileno de 15 mL, vedados com *parafilm*, e foram armazenados em freezer com temperatura monitorada em −26 °C. Antes do armazenamento, uma alíquota de cada controle de qualidade foi analisada no dia do preparo, considerada tempo = 0 (zero), para então ser comparada com as amostras a serem analisadas nos intervalos de 7, 14 e 30 dias, de acordo com as condições que se pretendia armazenar as amostras reais.

As amostras armazenadas podem ser consideradas como estáveis desde que os DPR%, entre o valor do tempo = 0 comparado ao valor do tempo = teste, seja inferior a 15%. Na Tabela 7 são demonstrados os resultados quanto a estabilidade dos analitos armazenados em freezer −26 °C.

Na rotina laboratorial, é necessário o conhecimento da estabilidade dos analitos, nas condições de armazenamento disponíveis no laboratório. Neste experimento, os analitos permaneceram estáveis nas amostras a −26 °C, por um período máximo de 30 dias, exceto para o analito epoxiconazol, que apresentou estabilidade por um período de 14 dias, sendo esse considerado então o período máximo para a análise das amostras, no presente estudo.

Tabela 7 – Desvios Padrões relativos (%) do teste de estabilidade de amostras de urina em freezer com 7,14 e 30 dias de armazenamento

	7 dias (-26°C)			14 dias (-26°C)			30 dias (-26°C)		
Triazol	CQB	CQM	CQA	CQB	CQM	CQA	CQB	CQM	CQA
Ciproconazol	7,33	6,79	4,83	5,11	4,61	4,86	2,36	7,80	7,15
Epoxiconazol	12,40	11,33	10,97	7,40	11,05	8,44	34,07	20,94	21,44
Metconazol	7,87	6,69	5,91	4,96	2,69	5,05	11,22	7,87	6,11
Propiconazol	7,24	7,29	3,38	6,35	3,33	3,66	6,73	6,85	3,66
Triadimenol	8,50	10,93	6,15	11,65	7,55	7,10	10,60	10,50	6,61

Fonte: Do Autor.
Nota: CQB: 50 µg/L; CQM: 120 µg/L; CQA: 180 µg/L.

Portanto, a aplicação do bioindicador de dose interna de triazóis urinários por CG-MS teve como objetivo avaliar a intensidade de exposição do grupo exposto aos agrotóxicos e confirmar a ausência de exposição no grupo urbano. Ao incorporar a etapa de secagem em uma centrífuga concentradora/evaporadora a vácuo, o método desenvolvido por Machado *et al*. (2019) foi validado e de acordo com as diretrizes nacionais e internacionais para análise de amostras biológicas.

A Tabela 8 apresenta a faixa de concentração dos triazóis detectados no grupo rural. Nenhuma amostra do grupo urbano apresentou sinais acima do limite de detecção do método (2 µg/L para ciproconazol, metconazol e triadimenol; 5 µg/L para epoxiconazol e propiconazol), confirmando a ausência de exposição ocupacional aos agrotóxicos no grupo urbano .

Tabela 8 – Faixa de concentração de fungicidas triazóis urinários e normalização pela creatinina urinária

Triazóis detectados no grupo exposto	Faixa (µg/L)	Faixa (µg/g creatinina)
Homens		
Ciproconazol	< LIQ – 15,0	< LIQ – 9,6
Epoxiconazol	< LIQ – 70,0	< LIQ – 66,7*
Triadimenol	45,5	33,18
Mulheres		
Epoxiconazol	< LIQ – 40,2	< LIQ – 89,3*

Fonte: Do Autor.
Nota: Limite de quantificação (LIQ); LIQ = 10 µg/L para ciproconazol e triadimenol; 30 µg/L para epoxiconazol. *valor-p significativo e obtido pelo teste de Mann-Whitney, com 5% de significância.

A coleta das amostras de urina foi realizada para coincidir com a época do ano

em que os agricultores estão aplicando os triazóis, entre os meses de novembro e março. Dessa maneira, a coleta das amostras foi realizada após a aplicação dos fungicidas na lavoura, dentro do período de maior taxa de excreção (Oestreich, Schmid e Schlatter, 1997).

Conforme antecipado pela observação dos dados obtidos pelo questionário, o fungicida mais frequentemente detectado foi o epoxiconazol, que esteve presente em 86% das amostras de homens e mulheres residentes em áreas rurais. Estes dados estão em concordância que o epoxiconazol é o fungicida triazol mais comumente utilizado em plantações de café na região (Machado *et al.*, 2019). Ciproconazol foi detectado e quantificado em 12% das amostras, exclusivamente em amostras de urina masculina. Entre essas amostras, 5% apresentaram detecção simultânea de epoxiconazol e ciproconazol. Além disso, uma amostra apresentou a presença de triadimenol na concentração de 45,5 µg/L, apesar deste fungicida não ter sido relatado no questionário.

As concentrações relativas de fungicidas triazóis na urina foram analisadas e comparadas estatisticamente, após a normalização com os níveis de creatinina urinária. Esta abordagem avalia a adequação da carga corporal como um bioindicador de exposição, de maneira semelhante a outros indicadores biológicos. Os valores de concentração de epoxiconazol, quando normalizados pela creatinina urinária, mostraram-se significativos pelo teste de Mann-Whitney (valor-p = 0,0406). Este analito foi selecionado devido à sua alta frequência de detecção no grupo exposto e à sua presença tanto em homens quanto em mulheres, o que proporciona maior confiabilidade estatística. Portanto, a normalização pela creatinina urinária demonstrou ser aplicável neste contexto, ajudando a corrigir possíveis variações nas concentrações de elementos que podem ser influenciadas pelo volume de urina (Aguera *et al.*, 2022).

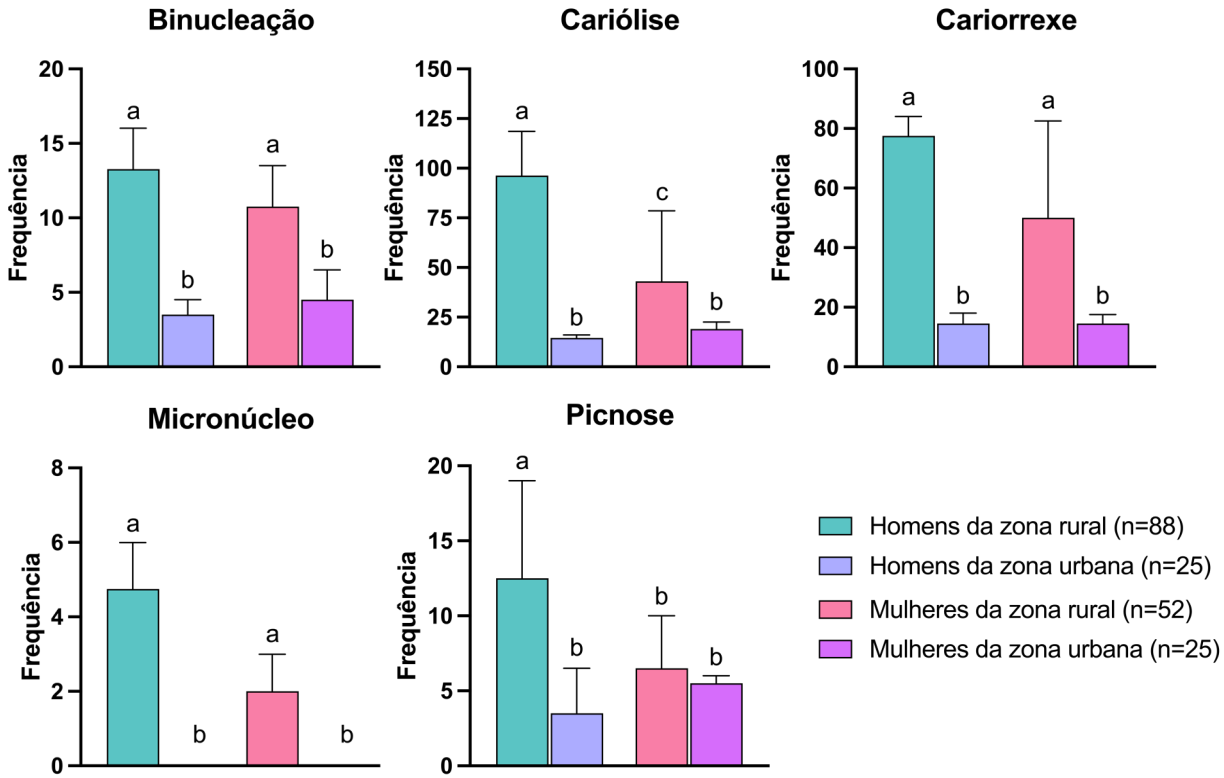
5.3 BIOMARCADORES DE GENOTOXICIDADE E DE ESTRESSE OXIDATIVO

O ensaio do citoma em células de mucosa bucal é um ensaio minimamente invasivo, que contribui para o estudo do dano ao DNA, a partir da verificação de parâmetros relacionados a instabilidade cromossômica, morte celular e potencial regenerativo do tecido da mucosa bucal (Machado, 2018). Os biomarcadores deste ensaio têm sido associados ao aumento do risco de envelhecimento acelerado, câncer

e doenças neuro-degenerativas (Benedetti *et al.*, 2013; Machado e Martins, 2018). Foram avaliados biomarcadores associados aos efeitos de genotoxicidade (micronúcleo), defeitos na citocinese (binucleação), e morte celular por apoptose e necrose (cariólise, cariorrexe e picnose) (Borba *et al.*, 2019; Thomas *et al.*, 2009).

A Figura 7 apresenta a frequências dos biomarcadores do ensaio do citoma encontrados nos grupos da zona rural e zona urbana, avaliados pela análise de variância pelo teste de Kruskall-Wallis, com 5% de significância.

Figura 7 – Frequência dos biomarcadores do ensaio do citoma



Fonte: Do Autor.

Nota: Valores de frequência expresso em mediana e 95% de intervalo de confiança. *valor-p obtido através do teste de Kruskal-Wallis, com 5% de significância. Valores com letras iguais não apresentaram diferença significativa (Teste de Dunn, 5% de significância).

É possível observar maior frequência de alterações encontradas no grupo dos homens da zona rural que aplicam os agrotóxicos quando comparado às mulheres que vivem na zona rural e entre os grupos da zona urbana. Estes resultados estão em concordância com os resultados de Silvério *et al*. (2017) que foi realizado anteriormente na mesma região.

Em relação a comparação entre os grupos expostos, os homens apresentam aumento significativo da frequência de cariólise e picnose, indicando efeitos de morte

celular por apoptose e necrose mais acentuado do que as mulheres da zona rural.

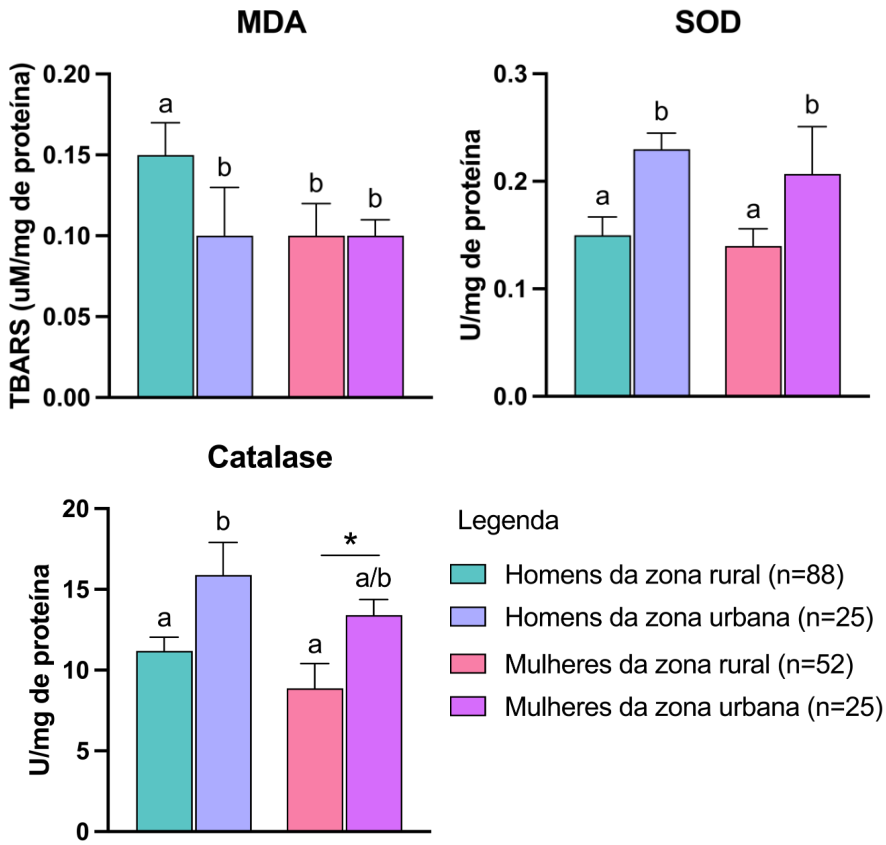
Em determinadas condições de exposição, os xenobióticos, como os agrotóxicos, induz a geração de espécies reativas, que supera os antioxidantes produzidos endogenamente e o sistema defesa enzimático, levando à morte celular progressiva (Memudu e Dongo, 2023). E a ocorrência de morte celular por apoptose e necrose são frequentemente sincronizadas com o estresse oxidativo (Liu *et al.*, 2021).

O equilíbrio entre a produção de oxidantes, espécies reativas de oxigênio e nitrogênio e a defesa antioxidante do corpo desempenha um papel importante na manutenção da homeostase celular e tecidual em humanos (Simicic, Cudalbu e Pierzchala, 2022).

Poucos estudos avaliaram os efeitos oxidativos dos triazóis. Othmène *et al*. (2020) investigaram os efeitos da exposição subcrônica ao fungicida triazol tebuconazol em ratos machos adultos, avaliando biomarcadores de estresse oxidativo e alterações histopatológicas no tecido cardíaco. Os resultados mostraram que o tratamento com tebuconazol levou ao aumento dos níveis de MDA, enquanto SOD e catalase inicialmente aumentaram em doses de 0,9, 9 e 27 mg/kg-pc, mas diminuíram com uma dose de 45 mg/kg-pc, alterando o equilíbrio oxidativo e, consequentemente, danificando o tecido cardíaco.

Para avaliar o nível de estresse oxidativo nos grupos estudados, a peroxidação lipídica sérica foi medida pela quantificação de substâncias reativas ao TBARS que reflete indiretamente a produção de MDA. As enzimas SOD e catalase fazem parte do mecanismo de defesa antioxidante, trabalhando para neutralizar o excesso de produção de espécies oxidantes. A Figura 8 apresenta a análise de variância usando o teste de Kruskal-Wallis para MDA, SOD e catalase nos grupos da zona rural e urbana, estratificados por sexo.

Figura 8 – Comparação dos biomarcadores de estresse oxidativo



Fonte: Do Autor.

Nota: Valores expressos em mediana e 95% de intervalo de confiança. *valor-p obtido através do teste de Kruskal-Wallis, com 5% de significância. Valores com letras iguais não apresentaram diferença significativa (Teste de Dunn, 5% de significância).

Os dados indicam um nível mais elevado de peroxidação lipídica em homens da zona rural em comparação com homens da zona urbana, assim como em comparação com mulheres de ambos os grupos. Em relação ao sistema de defesa enzimático estudado, o grupo dos aplicadores de agrotóxicos tiveram níveis reduzidos quando comparado com o grupo de homens da zona urbana, indicando um desequilíbrio e uma situação de estresse oxidativo. Da mesma forma, as mulheres da zona rural, expostas ambientalmente aos agrotóxicos, também apresentaram os biomarcadores de defesa reduzidos quando comparado com o grupo das mulheres da zona urbana. No entanto, elas não apresentaram produção significativa de MDA, indicando que não estavam em estado de estresse oxidativo, o que poderia explicar os níveis reduzidos do sistema de defesa enzimático.

Os dados obtidos estão em concordância com outros estudos que avaliaram exposição ocupacional aos agrotóxicos. Abbasi-Jorjandi *et al*. (2020) e Surajudeen *et al*. (2014) mostraram diminuição significativa de atividade de SOD e catalase,

respectivamente, e níveis elevados de MDA em trabalhadores que aplicavam inseticidas organofosforados. No entanto, o estudo realizado por Hunderaki, Suryakar e Rathi (2013), que também avaliou a exposição ocupacional a organofosforados, mostrou níveis elevados de MDA no grupo exposto em comparação com o grupo de controle, mas observou um aumento nas atividades da SOD, catalase e glutationa peroxidase (GPx), sugerindo uma resposta adaptativa ao dano oxidativo causado pela exposição a organofosforados.

No entanto, ainda poucos estudos exploraram os desequilíbrios redox e biomarcadores de estresse oxidativo em brasileiros expostos aos agrotóxicos (Jacobsen-Pereira *et al.*, 2018; Nunes *et al.*, 2024). Jacobsen-Pereira *et al*. (2018) observaram um aumento na peroxidação lipídica e efeitos genotóxicos relacionados em trabalhadores rurais expostos aos agrotóxicos, mas não observaram uma mudança significativa nas atividades de CAT entre os grupos expostos e não expostos.

É possível observar que os resultados apresentados na Figura 8 podem estar associados e, talvez explicar, o perfil de variabilidade encontrado nas frequências dos biomarcadores do ensaio do citoma apresentado na Figura 7. Também existe uma relação entre a exposição ocupacional aos agrotóxicos organofosforados que demostraram relação entre o desequilíbrio oxidativo e genotoxicidade. Zepeda-Arce *et al*. (2017) encontraram uma diminuição marginalmente significativa nas atividades da SOD e catalase entre os trabalhadores que aplicavam esses agrotóxicos e observaram uma correlação positiva entre os parâmetros de danos ao DNA, medidos pelo ensaio do cometa, e os níveis de MDA.

5.4 ANÁLISE DOS NÍVEIS PLASMÁTICOS DE ÁCIDOS BILIARES E ENZIMAS HEPÁTICAS

5.4.1 Método para análise de ácidos biliares plasmáticos por UHPLC-MS/MS

A Tabela 9 apresenta as informações químicas dos ácidos biliares analisados.

Tabela 9 – Informações químicas dos ácidos biliares

Ácidos biliares	Formula molecular	Radical 1	Radical 2	Radical 3	Radical 4
CA	$C_{24}H_{40}O_5$	OH	H	OH	OH
DCA	$C_{24}H_{40}O_4$	H	H	OH	OH
GCA	$C_{26}H_{43}NO_6$	OH	H	OH	glicina
GDCA	$C_{26}H_{43}NO_5$	H	H	OH	glicina
TCA	$C_{26}H_{45}NO_7S$	OH	H	OH	taurina
TDCA	$C_{26}H_{45}NO_6S$	H	H	OH	taurina

Fonte: Silveira *et al*., (2022).
Nota: CA: ácido cólico, DCA: ácido deoxicólico, GCA: ácido glicocólico, GDCA: ácido glicodeoxicólico, TCA: ácido taurocólico, TDCA: ácido taurodeoxicólico.

A Tabela 10 apresenta os parâmetros espectrométricos para análise de ácidos biliares por UHPLC-MS/MS.

Tabela 10 – Parâmetros espectrométricos dos ácidos biliares

Ácidos biliares	Massa molecular (g/mol)	$[M–H]^-$ (*m*/*z*)*	Q3 (V)	Tempo de retenção (minutos)
Ácido cólico	408,57	407,20	26	10,3
Ácido deoxicólico	392,57	391,30	25	13,6
Ácido glicocólico	465,63	464,30	30	7,9
Ácido glicodeoxicólico	449,63	448,30	30	9,4
Ácido taurocólico	515,70	514,35	36	7,5
Ácido taurodeoxicólico	499,71	498,30	22	8,5

Fonte: Silveira *et al*. (2022).
Nota: *Íon monitorado em modo SIM (*Selected Ion Monitoring)*. CA: ácido cólico, DCA: ácido deoxicólico, GCA: ácido glicocólico, GDCA: ácido glicodeoxicólico, TCA: ácido taurocólico, TDCA: ácido taurodeoxicólico. Q3: Quadrupolo 3.

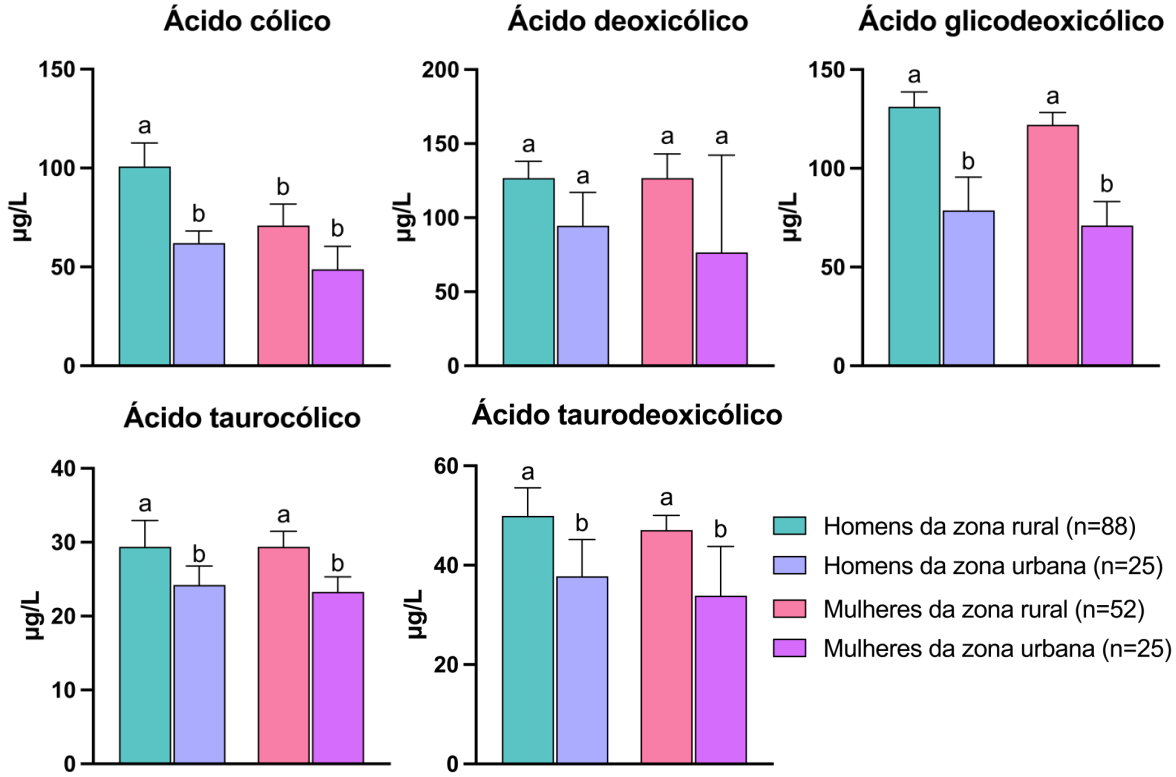
A faixa linear, com exatidão e precisão, obtida para os ácidos biliares CA, DCA e GDCA foi de 10 a 500 µg/L, enquanto a do TCA e TDCA foi de 25 a 500 µg/L, de acordo com as diretrizes da ANVISA (BRASIL, 2012) e FDA (FDA, 2018). Apenas o GCA não apresentou linearidade satisfatória, diferente do estudo de Machado *et al*. (2021) e de Luo *et al*. (2018), em que este ácido apresentou resultados potenciais como bioindicador de efeito.

5.4.2 Análise dos níveis de ácidos biliares e enzimas hepáticas

A análise dos ácidos biliares como um bioindicador de efeito é crucial na busca de um método para identificar os riscos e reverter potenciais danos hepáticos causados por fungicidas triazóis. Isto é particularmente importante dada a evidência de hepatotoxicidade associada a estes agrotóxicos (Ekman *et al.*, 2006; Goetz *et al.*, 2006; Heise *et al.*, 2015; Machado *et al.*, 2021; Tully *et al.*, 2006).

Além disso, estudos relataram concentrações significativamente mais altas de ácidos biliares em pacientes com insuficiência hepática em comparação com indivíduos saudáveis. Consequentemente, os ácidos biliares foram investigados como potenciais biomarcadores de danos hepáticos (Luo *et al.*, 2018; Sugita *et al.*, 2015). A Figura 9 apresenta a análise de variância usando o teste de Kruskal-Wallis para medidas de CA, DCA, GDCA, TCA e TDCA nos grupos rural e urbano, estratificados por sexo.

Figura 9 – Comparação dos níveis plasmáticos de ácidos biliares



Fonte: Do Autor.

Nota: Valores expressos em mediana e 95% de intervalo de confiança. **p-valor* obtido através do teste de Kruskal-Wallis, com 5% de significância. Valores com letras iguais não apresentaram diferença significativa (Teste de Dunn, 5% de significância).

Os dados indicam um aumento significativo nos ácidos biliares entre os homens e mulheres do grupo exposto, exceto para DCA onde não se observou diferença de dosagem entre os grupos. Os níveis de CA foram elevados apenas nos homens do grupo exposto, apresentando igualdade estatística quando comparadas as mulheres da zona rural e urbana. No entanto, os biomarcadores GDCA, TCA e TDCA exibiram diferença estatisticamente significativa entre residentes de áreas rurais e urbanas.

Estudos têm mostrado consistentemente que os ácidos biliares estão presentes em níveis de concentração plasmática mais altos em pacientes com danos hepáticos comparados a indivíduos saudáveis (Bathena *et al.*, 2015; Luo *et al.*, 2018; Trottier *et al.*, 2011; Woolbright *et al.*, 2014). Vale ressaltar que em seu estudo, Luo *et al*. (2018) compararam especificamente indivíduos saudáveis com pacientes hospitalizados que sofreram vários casos de lesão hepática.

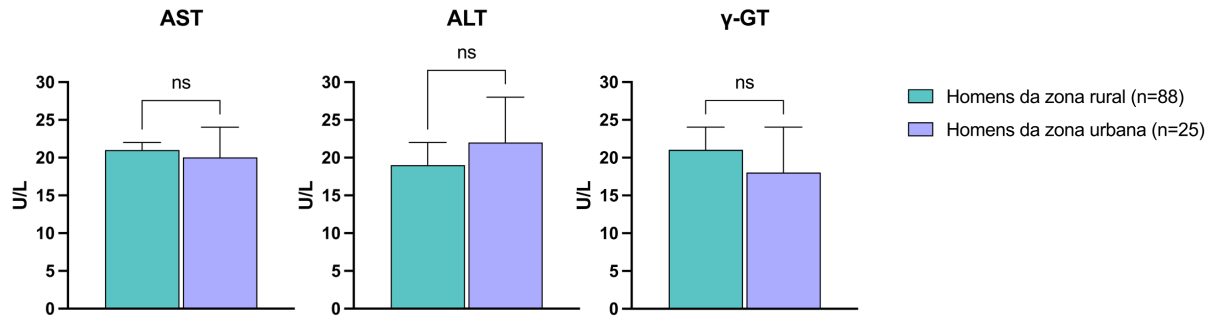
Dentre os ácidos biliares estudados, aqueles conjugados com glicina apresentaram as maiores concentrações. Isso pode ser atribuído à natureza hidrofóbica desses ácidos, já que a conjugação reduz sua hidrofobicidade (Ashby *et al.*, 2018; Luo *et al.*, 2018; Machado *et al.*, 2021). Os ácidos biliares não conjugados, por outro lado, são mais hidrofóbicos e citotóxicos, levando a danos mitocondriais e ruptura da membrana celular, podendo causar necrose e apoptose em hepatócitos devido à geração de radicais livres (Bechmann *et al.*, 2013; Guicciardi *et al.*, 2013; Jang *et al.*, 2012; Luo *et al.*, 2018). Outros estudos (Goetz and Dix, 2009; Machado *et al.*, 2021) relatam que transportadores envolvidos no metabolismo de esteroides, colesterol, aminoácidos e absorção de ácidos biliares foram super-regulados pela exposição a triazóis, indicando um aumento na captação desses agrotóxicos pelos hepatócitos, aumentando assim o dano causado por eles e a subsequente excreção de seus metabólitos. Embora a dieta possa influenciar a concentração de ácidos biliares até certo ponto, o impacto dos fatores dietéticos é mínimo comparado ao aumento associado a danos hepáticos e toxicidade (Bathena *et al.*, 2015).

Concentrações mais baixas de DCA podem ser observadas em pacientes hospitalizados com insuficiência hepática (Luo *et al.*, 2018). Além disso, uma redução significativa nos níveis de DCA pode estar associada a doenças do trato biliar (Sugita *et al.*, 2015). As doenças hepáticas colestáticas são caracterizadas por um aumento nos ácidos biliares primários e uma diminuição nos ácidos biliares secundários, como o DCA (Humbert *et al.*, 2012; Sugita *et al.*, 2015; Trottier *et al.*, 2011, 2012).

No dano hepático, a membrana celular dos hepatócitos é rompida, portanto, as

enzimas AST, ALT e γ-GT são liberadas na corrente sanguínea, causando aumento nos níveis plasmáticos e indicando a presença de dano hepático (Masubuchi *et al.*, 2016; Woreta e Alqahtani, 2014). Como alterações foram observadas tanto nos biomarcadores de estresse oxidativo quanto nos ácidos biliares entre os homens do grupo exposto, as enzimas hepáticas dos voluntários masculinos dos grupos exposto e não exposto foram medidas e apresentadas na Figura 10.

Figura 10 – Comparação das enzimas hepáticas de voluntários masculinos da zona rural e urbana



Fonte: Do Autor.
Nota: Valores expressos em mediana e 95% de intervalo de confiança. *valor-p obtido através do teste de Mann-Whitney, com 5% de significância.

Os dados mostram que não houve diferença estatisticamente significativa entre os grupos pelo teste de Mann-Whitney, já que apresentaram valores muito semelhantes na medição dos três parâmetros bioquímicos.

Os voluntários expostos aos agrotóxicos mostraram menor consumo de álcool (27 %), comparado ao grupo não exposto da área urbana (62 %), conforme descrito anteriormente na Tabela 1 (seção 5.1). Essa diferença no consumo de álcool é crítica para considerar como potencial fator confundidor na análise de biomarcadores. No entanto, os resultados significativos relacionados ao estresse oxidativo e ao aumento dos ácidos biliares parecem não ser afetados por essa variável. É importante notar que o grupo urbano, que não foi exposto ocupacionalmente aos agrotóxicos, teve a maior taxa de consumo de álcool, sublinhando a confiabilidade desses resultados.

No contexto dos hábitos de fumar, a diferença foi mínima, com os grupos exposto e não exposto mostrando 15% e 16%, respectivamente (Tabela 1 seção 5.1), indicando que essa variável não influencia significativamente o efeito da exposição a agrotóxicos nos biomarcadores estudados. Essas tendências são consistentes com outras pesquisas na área (Costa *et al.*, 2023; Silvério *et al.*, 2017)

Portanto, é possível inferir que os biomarcadores estudados estão demonstrando alterações precoces e possivelmente reversíveis, uma vez que ainda não foram observadas mudanças significativas nos parâmetros bioquímicos utilizados para o diagnóstico de doenças hepáticas.

5.5 PARÂMETROS BIOQUÍMICOS E NÍVEIS DE HORMÔNIOS ESTEROIDES

A distribuição e comparação dos níveis de colesterol, HDL, LDL, VLDL, triglicerídeos e glicose por meio do teste de Kruskal-Wallis estão descritas na Tabela 11.

Tabela 11 – Distribuição e comparação múltipla pelo teste de Kruskal-Wallis dos parâmetros bioquímicos

Parâmetro bioquímico	Grupo	Média	DP	Mín.	P25	Mediana	P75	Máx.	K.W. valor-p	Valor de referência (mg/dL)
Colesterol*	Homens rural	181,4	39,5	96,0	163,8	178,5	198,2	351,0	0,046	< 190,0
	Mulheres rural	186,9	37,3	109,0	168,0	180,5	213,5	311,0		
	Homens urbano*	164,6	33,6	90,0	144,0	169,0	181,0	239,0		
HDL*	Homens rural	50,8	11,3	29,0	43,0	49,0	59,0	89,0	0,008	> 40,0
	Mulheres rural	54,4	10,8	31,0	47,8	54,0	62,5	82,0		
	Homens urbano*	45,8	11,0	20,0	38,0	42,0	57,0	63,0		
LDL	Homens rural	107,7	35,2	15,8	90,4	107,8	121,3	258,0	0,492	< 130,0
	Mulheres rural	108,1	34,9	25,6	90,1	106,1	127,7	229,8		
	Homens urbano	100,6	29,8	47,4	84,6	100,8	111,0	173,6		
VLDL	Homens rural	23,0	13,5	6,0	13,4	18,4	29,7	75,2	0,198	< 40,0
	Mulheres rural	24,4	12,5	7,8	15,5	20,4	28,9	56,8		
	Homens urbano	18,1	4,6	6,6	15,2	17,0	22,6	26,2		
Triglicerídeos	Homens rural	114,8	67,5	30,0	67,0	92,0	148,5	376,0	0,198	< 175,0
	Mulheres rural	122,1	62,4	39,0	77,8	102,0	144,5	284,0		
	Homens urbano	90,7	23,1	33,0	76,0	85,0	113,0	131,0		
Glicose*	Homens rural*	99,4	17,9	75,0	91,8	97,5	102,0	240,0	0,008	< 99,0
	Mulheres rural	98,7	23,0	65,0	86,8	92,5	100,5	194,0		
	Homens urbano	92,1	6,2	80,0	87,0	92,0	95,0	111,0		

Fonte: Do Autor.
Nota: Resultados significativos com base no teste de Kruskal-Wallis (K.W.) e no teste de Dunn como post hoc com 5% de significância.

A Tabela 12 detalha os resultados das comparações realizadas pelo teste de Dunn como post hoc dos parâmetros bioquímicos.

Tabela 12 – Comparações pelo teste de Dunn dos parâmetros bioquímicos

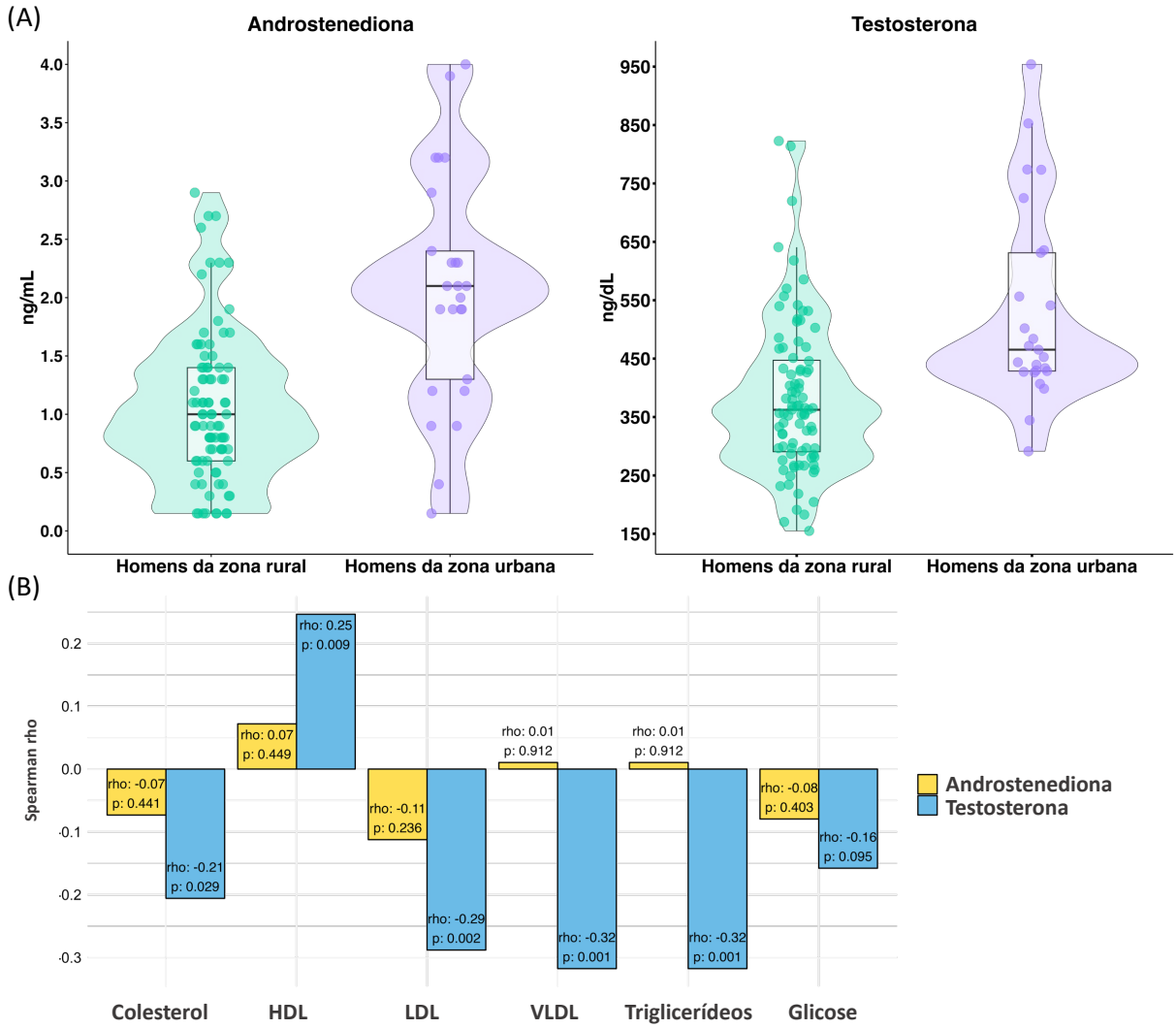
Parâmetros bioquímicos	K.W. valor-p	Comparação	Teste Dunn valor-p	Significância
Colesterol	0,046	Homens rural x Homens urbano	0,087	n.s.
		Homens rural x Mulheres rural	0,486	n.s.
		Homens urbano x Mulheres rural	0,020	*
HDL	0,008	Homens rural x Homens urbano	0,093	n.s.
		Homens rural x Mulher rural	0,103	n.s.
		Homens urbano x Mulheres rural	0,003	*
LDL	0,492	N/A	N/A	n.s.
VLDL	0,198	N/A	N/A	n.s.
Triglicerídeos	0,198	N/A	N/A	n.s.
Glicose	0,008	Homens rural x Homens urbano	0,009	*
		Homens rural x Mulheres rural	0,043	*
		Homens urbano x Mulheres rural	0,490	n.s.

Fonte: Do Autor.
Nota: Resultados significativos com base no teste de Kruskal-Wallis (K.W.) e no teste de Dunn como post hoc com 5% de significância. N/A: Não aplicável.

O teste de Kruskal-Wallis não revelou diferenças significativas entre os grupos nos níveis de LDL, VLDL e triglicerídeos. Em contraste, embora a análise não paramétrica de variância para os níveis de colesterol e HDL não tenha indicado diferenças significativas entre agricultores e homens urbanos, os níveis nas mulheres rurais foram significativamente diferentes para estes marcadores quando comparados com o grupo de homens urbanos (valor-p do teste de Dunn 0,020 e 0,003, respectivamente). Em relação aos níveis de glicose, foi observada uma diferença significativa no grupo de agricultores em comparação com homens urbanos e mulheres rurais (valor-p do teste de Dunn 0,009 e 0,043, respectivamente).

Na comparação dos níveis hormonais entre os homens do grupo rural exposto e os da área urbana, observamos reduções significativas nos níveis de testosterona e androstenediona entre os voluntários rurais. A Figura 11 ilustra essa comparação para ambos os hormônios esteroides, empregando o teste de Mann-Whitney e a associação com os resultados dos parâmetros bioquímicos em voluntários do sexo masculino, utilizando o teste de correlação de Spearman.

Figura 11 – Comparação de hormônios esteroides masculinos e associação com parâmetros bioquímicos



Fonte: Do Autor.

Nota: (A) Medições de androstenediona e testosterona total. *valor-p obtido usando o teste de Mann-Whitney, com 5% de significância. Na androstenediona dos homens da zona urbana, um ponto *outlier* (8,4 ng/mL) foi removido. (B) Correlação de Spearman entre hormônios esteroides e parâmetros bioquímicos. Homens da zona rural n = 88; Homens da zona urbana n = 25.

A análise de correlação de Spearman revelou relação significativa entre os níveis de testosterona e todos os parâmetros avaliados (valor p < 0,05). Correlações inversas (rho < 0) foram observadas, indicando que à medida que os níveis de testosterona diminuíam, os níveis de colesterol, LDL, VLDL, triglicerídeos e glicose tendiam a aumentar. Por outro lado, uma correlação direta (rho > 0) com o HDL sugeriu que à medida que os níveis de testosterona aumentavam, os níveis de HDL aumentavam. Em contraste, as correlações entre os níveis de androstenediona e qualquer um dos parâmetros analisados não foram estatisticamente significativas.

Os fungicidas triazóis são reconhecidos por seu mecanismo de ação como

potentes inibidores da CYP51 (McLean *et al.*, 2006). Em humanos, a CYP51 desempenha um papel crucial no processo de desmetilação do colesterol, que é fundamental para a produção de ácidos biliares, mineralocorticoides, glicocorticoides e hormônios esteroides sexuais (Lepesheva e Waterman, 2011). Dado esse papel, foi relatado em estudos anteriores o potencial dos triazóis em desregular as funções endócrinas ao interferir na síntese de esteroides (Lv *et al.*, 2017; Machado *et al.*, 2021; Poulsen *et al.*, 2015). Para investigar esses efeitos, análises hormonais foram realizadas em amostras de soro coletadas de voluntários do sexo masculino residentes em ambos ambientes rurais e urbanos. O foco em voluntários do sexo masculino é particularmente pertinente ao contexto agrícola do sul de Minas Gerais, Brasil, onde a aplicação de agrotóxicos é predominantemente uma atividade masculina, alinhando-se assim com o primeiro objetivo do estudo de avaliar o impacto da exposição ocupacional aos agrotóxicos neste grupo demográfico.

Os resultados indicam que os níveis de testosterona nos agricultores foram significativamente mais baixos do que nos homens urbanos (Mann-Whitney, valor $p < 0,05$). Entretanto, seu valor mediano e intervalo de confiança de 95% permaneceram acima do valor de referência estabelecido de 175,0 ng/dL para esse hormônio. Da mesma forma, os níveis de androstenediona seguiram um padrão comparável, mas com as medições do grupo de agricultores aproximando-se do valor de referência inferior de 0,6 ng/mL. Notavelmente, 23 amostras dos agricultores foram iguais ou abaixo do valor de referência para este hormônio, contrastando com apenas 2 amostras no grupo urbano que se situaram abaixo deste limiar.

Para investigar melhor as alterações hormonais dos esteroides, também foram medidos parâmetros bioquímicos. Foram observadas diferenças no colesterol e no HDL entre o grupo de mulheres rurais e os homens urbanos. Além disso, as diferenças notáveis nos níveis de glicose entre os agricultores e os outros grupos sublinham as consequências metabólicas da exposição prolongada aos agrotóxicos. Considerando a importância do metabolismo da glicose na saúde geral, essas diferenças podem refletir um indicador precoce de interrupção da homeostase metabólica entre o grupo de agricultores (Chen *et al.*, 2019).

A análise de correlação de Spearman revelou associações significativas entre os níveis de testosterona e marcadores bioquímicos. Níveis reduzidos de testosterona, caracterizados por aumento de massa gorda, redução da sensibilidade à insulina, tolerância diminuída à glicose, triglicerídeos e colesterol elevados e HDL

baixo refletem a síndrome metabólica e o diabetes tipo 2, que contribuem para o risco cardiovascular (Kelly e Jones, 2013). Este padrão ressalta a influência potencial da exposição ocupacional e ambiental aos fungicidas nos níveis de testosterona e suas implicações metabólicas mais amplas.

5.6 CÁLCULOS DE RISCO

Os resultados do cálculo da EDI e os valores utilizados são apresentados na Tabela 13. A EDI foi calculada para concentrações urinárias de triazóis (µg/g crea.) variando desde valores acima do LD e abaixo do LIQ do método, incluindo percentil 25, percentil 50 (mediana), percentil 75 e valor máximo quantificado.

Os dados demonstram que a EDI do epoxiconazol é superior em comparação com outros triazóis, principalmente devido à sua elevada concentração nas amostras de urina coletadas e ao seu baixo valor de F (0,17) (EFSA, 2008a). Além disso, a IDA para epoxiconazol é menor, sendo de 0,003 mg/kg-pc/dia (ANVISA, 2024), em contraste com os outros triazóis detectados, que têm valores de IDA de 0,01 mg/kg-pc/dia e 0,05 mg/kg-pc/dia, de acordo com com a legislação brasileira (ANVISA, 2024) e a Base de Dados de Pesticidas da UE (EC, 2023) para o ciproconazol e o triadimenol, respectivamente. Essa discrepância resulta em valores mais altos de HQ quando se compara epoxiconazol com ciproconazol e triadimenol, conforme descrito na Tabela 13.

Tabela 13 – *Estimated Daily Intake* (EDI, µg/kg-pc/dia) e *Hazard Quotient* (HQ) dos fungicidas triazóis detectados

EDI	< LIQ[†]	P25	Mediana	P75	Max	IDA
Homens da zona rural						
Ciproconazol	0,169	0,216	0,235	0,281	0,572	10,0
Epoxiconazol	0,534	0,785	1,08	2,96	6,31	3,0
Triadimenol	-	-	-	-	1,07	50,0
Mulheres da zona rural						
Epoxiconazol	0,657	1,01	1,89	2,54	8,77	8,0
HQ						
Homens da zona rural						
Ciproconazol	0,017	0,022	0,024	0,028	0,057	
Epoxiconazol	0,158	0,261	0,359	0,765	2,103	
Triadimenol	-	-	-	-	0,021	
Mulheres da zona rural						
Epoxiconazol	0,163	0,336	0,629	0,846	2,922	

Fonte: Do Autor.

Nota: [†]Concentração de triazóis na urina (µg/g creatinina) abaixo do limite de quantificação (LIQ); LIQ = 10 µg/L para ciproconazol e triadimenol; 30 µg/L para epoxiconazol; percentil 25, percentil 50 (mediana), percentil 75 e valor máximo detectado. IDA = Ingestão Diária Aceitável (µg/kg-pc/dia).

Nos homens do grupo exposto, o HQ excedeu uma unidade no valor máximo detectado de triazol urinário encontrado no grupo masculino (2.103), indicando risco significativo à saúde com base nos cálculos realizados. Os resultados anteriores apresentados deste estudo corroboram esses dados, revelando potenciais efeitos resultantes da exposição aos agrotóxicos, principalmente os fungicidas triazóis, como evidenciado pelos resultados dos biomarcadores avaliados. Esses efeitos são particularmente evidentes na região cafeeira, onde esses fungicidas são amplamente utilizados (Machado *et al.*, 2021).

Além disso, vale destacar o HQ calculado para as mulheres do grupo exposto, que ultrapassou uma unidade (2,922), indicando alto risco à saúde. Essa observação é atribuída ao valor máximo detectado de triazol urinário encontrado no grupo feminino, proveniente de uma amostra com alta concentração de epoxiconazol (40,2 µg/L). Quando normalizado pela creatinina urinária (0,45 g/L), resultou em um valor de concentração urinária significativamente maior, consequentemente maior valor de EDI e HQ. Isso destaca a importância de considerar variações potenciais nas concentrações das substâncias, que podem ser influenciadas pelo volume de urina (Aguera *et al.*, 2022).

Com relação aos valores de IDA utilizados, caso fosse considerada a IDA indicada pela Comissão Européia para o epoxiconazol, sendo 0,008 mg/kg-pc/dia (EC,

2023; EFSA, 2015), não ultrapassaria uma unidade no HQ estimado para os homens, e sim apenas para o valor máximo de triazóis detectado no grupo de mulheres expostas. Assim, indicando que mesmo no pior cenário possível, maior intensidade de exposição representada pelo maior valor de triazol urinário encontrado, os aplicadores de agrotóxicos teriam seu risco subestimado. Também é importante destacar que, atualmente, tanto o epoxiconazol, quanto o ciproconazol e o triadimenol não têm validade de aprovação na Europa, de acordo com o banco de dados de agrotóxicos da União Europeia (EC, 2023). Justificando-se, assim, a importância da regionalização deste estudo, utilizando-se a IDA disponibilizada pela autoridade brasileira (ANVISA, 2024) na qual é permitido o uso desses fungicidas. Além disso, os cálculos de risco realizados são mais representativos ao refletir as condições reais de exposição da população estudada. Essas condições incluem a falta de utilização ou uso incompleto de EPIs pelos agricultores e a exposição ocupacional crônica aos fungicidas triazóis, conforme descrito na Figura 6 (seção 5.2.1).

Poucos estudos avaliaram o risco dos fungicidas triazóis resultantes da exposição ocupacional e ambiental. Em adultos e crianças tchecos, o tebuconazol, um fungicida triazol, foi detectado em quase todas as amostras de urina, indicando exposição principalmente impulsionada pela dieta e sugerindo um impacto menos significativo da exposição ambiental sem uma associação clara com a proximidade ou áreas agrícolas ao redor das residências, resultando em um menor risco (Šulc *et al.*, 2022). No entanto, nosso estudo revela descobertas contrastantes, pois tanto homens quanto mulheres que vivem em áreas rurais exibem níveis mais altos de exposição ambiental a fungicidas. Essa distinção é particularmente evidente nos resultados observados entre as mulheres que não estão expostas ocupacionalmente, e no pior cenário, amostra com a máxima concentração de triazol detectada, o HQ indica um maior risco para a saúde.

Estudos recentes utilizaram amostras biológicas para avaliar o risco potencial associado à exposição aos agrotóxicos em populações com características específicas. Por exemplo, na Espanha, metabólitos de agrotóxicos organofosforados, herbicidas e piretróides foram analisados em mães lactantes. Os resultados indicaram que fatores como a proximidade de atividades agrícolas, local de residência e a presença de jardins ou plantas nas casas contribuíram significativamente para os níveis urinários de metabólitos de agrotóxicos (Fernández, Pardo, Adam-Cervera, *et al.*, 2020). Além disso, outro estudo conduzido na Espanha (Fernández, Pardo,

Corpas-Burgos, *et al.*, 2020) avaliou o risco de exposição aos agrotóxicos organofosforados, herbicidas e piretróides em crianças, enquanto um estudo em Portugal focou na exposição ao glifosato (Ferreira *et al.*, 2021). Embora nenhum desses estudos tenha fornecido evidências de riscos à saúde, eles não aplicaram potenciais biomarcadores de efeito para investigar alterações precoces da exposição aos agrotóxicos, como realizadas neste estudo.

5.7 TOXICOLOGIA COMPUTACIONAL

A ferramenta *Search* foi usada para extrair dados sobre os três triazóis detectados: epoxiconazol, ciproconazol e triadimenol, e os três ingredientes ativos, azoxistrobina, piraclostrobina e tiametoxam, dos produtos OPERA®, PrioriXtra® e Verdadero®, com uso relatado pelos agricultores. A Tabela 14 apresenta os agrotóxicos juntamente com as predições de exposição fornecidas pela EPA para estes compostos químicos.

O CASRN dos compostos químicos foi inserido automaticamente na ferramenta *Curve Surfer*, selecionando todos os conjuntos de dados cHTS disponíveis. O primeiro filtro aplicado foi por '*Call*', resultando em 755 (16,8%) ensaios classificados como ativos, 3.620 (80,4%) como inativos e 129 (2,9%) sinalizados como omitidos (devido a problemas de controle de qualidade ou outros problemas de interferência na tecnologia de ensaio). Os ensaios considerados '*Actives*' foram então escolhidos para posterior análise. Dado o grande número de ensaios *in vitro* ativos e a diversidade de *mechanistic targets* para estes agrotóxicos, juntamente com o desafio de representar graficamente estes resultados, foi criada uma nova categorização para os agrupamentos de *mechanistic targets* dos dados de cHTS. Esta categorização foi modelada de forma semelhante à estrutura existente na ferramenta *Curve Surfer* na janela de entrada de dados dos ensaios selecionados e está descrita na Tabela 15.

Tabela 14 – Agrotóxicos selecionados com base nos produtos relatados no questionário e suas predições de exposição dentro da ferramenta *Search* do ICE

Agrotóxico	CASRN	Produto	Classe	Predição de Exposição SEEM3 (mg/kg/day) [b]		
				P5	Median	P95
Azoxistrobina	131860-33-8	Priori Xtra	Fungicida estrobilurina	7,47E-09	1,16E-07	7,83E-05
Ciproconazol	94361-06-5	Verdadero/Priori Xtra	Fungicida triazol	2,67E-08	2,03E-07	2,60E-04
Epoxiconazol	133855-98-8	OPERA	Fungicida triazol	2,54E-09	1,36E-07	6,09E-05
Piraclostrobina	175013-18-0	OPERA	Fungicida estrobilurina	9,01E-09	1,04E-07	5,91E-05
Tiametoxam	153719-23-4	Verdadero	Inseticida Neonicotinoide	2,03E-08	4,74E-07	7,88E-05
Triadimenol	55219-65-3	Triazole detected [a]	Fungicida triazol	2,35E-08	6,80E-07	5,18E-04

Fonte: Do Autor.

Nota: [a] Triazol detectado por CG-MS em uma amostra, mas nenhum produto relatado pelos agricultores no questionário possui este triazol como ingrediente ativo. [b] Predições de exposição da EPA, dados coletados da ferramenta *Search* do ICE versão 4.0.2.

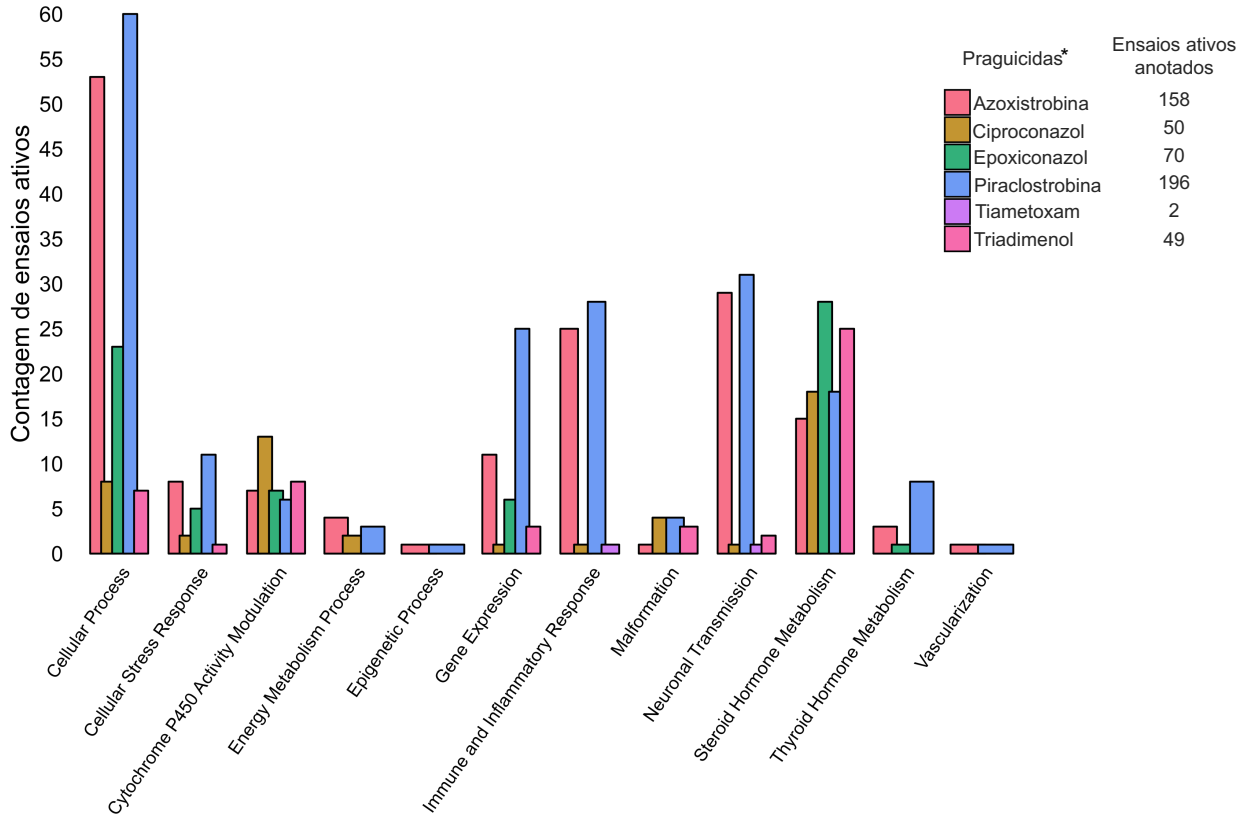
Tabela 15 – Categorização de *mechanistic targets* de ensaios ativos filtrados na ferramenta *Curve Surfer* do ICE

Cores	Categorias	*Mechanistic Targets* no ICE
	Cellular Process	Cell Cycle Cell Morphology Cellular Proliferation Cell Viability Extracellular Matrix Degradation
	Cellular Stress Response	DNA Damage p53 Modulation Oxidative Stress
	Cytochrome P450 Activity Modulation	Cytochrome P450 Activity Modulation Other Xenobiotic Response Transcription Factors
	Energy Metabolism Process	Energy Metabolism Process Mitochondrial Function
	Epigenetic Process	Histone Modification
	Gene Expression	Aryl Hydrocarbon Receptor Modulation Farnesoid X-activated Receptor Modulation Other Transcription Factors Other Developmental Signaling Transcription Factors RAR-related Orphan Receptor Modulation Retinoic Acid Receptor Modulation Retinoid X Receptor Modulation Vitamin D Modulation
	Immune and Inflammatory Response	Clotting Inflammation
	Malformation	Malformation
	Neuronal Transmission	Adenosine Receptor Modulation Dopamine Transporter Activity Modulation Ion Channel Activity Neurotransmission Opioid Receptor Modulation
	Steroid Hormone Metabolism	Androgen Receptor Modulation Aromatase Activity Modulation Cholesterol Transport Estrogen Biosynthesis and Metabolism Estrogen Receptor Modulation Glucocorticoid Biosynthesis and Metabolism Glucocorticoid Receptor Modulation Progestogen Biosynthesis and Metabolism Progesterone Receptor Modulation
	Thyroid Hormone Metabolism	Thyroid Receptor Modulation Sodium/Iodide Cotransporter
	Vascularization	Vascularization

Fonte: Do Autor.
Nota: Categorias e *mechanistic targets* mantidos em inglês para realizar o mapeamento das cores para corresponderem os dados originais extraídos do ICE.

A Figura 12 apresenta a distribuição dos *mechanistic targets* entre os ensaios ativos, identificados usando a ferramenta *Curve Surfer*.

Figura 12 – Distribuição dos *mechanistic targets* de ensaios ativos



Fonte: Do Autor.
Nota: *Pesticidas adicionais identificados na formulação de produtos utilizados por agricultores. Dados cHTS do ICE versão 4.0.2. Filtrados 230 ensaios ativos não anotados: Azoxistrobina = 79; Ciproconazol = 31; Epoxiconazol = 0; Piraclostrobina = 77; Tiametoxam = 4; Triadimenol = 39.

A ferramenta IVIVE emprega modelos PBTK, especificamente o pacote httk em R, para estimar o EAD com base na concentração de atividade dos ensaios ativos selecionados através de dosimetria reversa. Os ensaios *in vitro* ativos e o CASRN dos agrotóxicos foram inseridos automaticamente da *Curve Surfer* usando o botão '*Sent results to*' para a ferramenta IVIVE. Para representar ainda mais a categorização dos *mechanistic targets*, os dados de biomonitoramento humano e as predições de exposição SEEM3, os resultados da análise IVIVE foram exportados em um arquivo Excel do ICE. Depois disso, os ensaios *in vitro* foram codificados por cores usando R para ilustrar os *mechanistic targets* dos EADs e sua distribuição.

A Figura 13 compara os EADs orais, calculados a partir dos valores de ACC dos ensaios ativos *in vitro*, as EDI dos dados de biomonitoramento e as predições de exposição SEEM3.

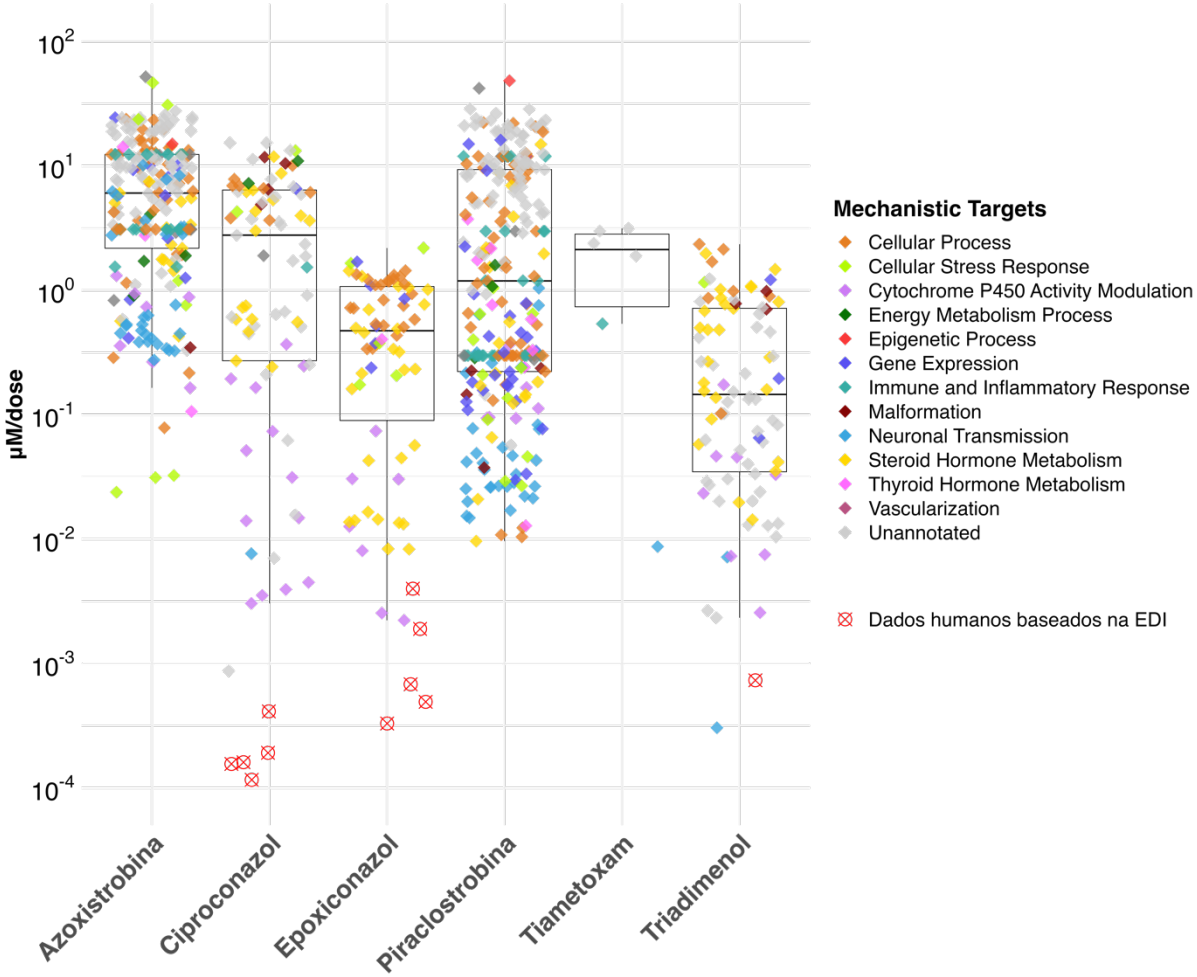
Figura 13 – Comparação das EADs orais derivadas de dados *in vitro* cHTS, dados do biomonitoramento humano (EDI) e predições de exposição SEEM3 nos 95 percentis

Fonte: Do Autor.

Nota: EAD: *equivalent administered dose*. EDI: *Estimated Daily Intake*. SEEM3: *Systematic Empirical Evaluation of Models*.

A Figura 14 apresenta os EADs de inalação, derivados utilizando o modelo de gás, em comparação com a EDI apenas dos agricultores, refletindo os dados de exposição ocupacional disponíveis (Figura 6, seção 5.2.1).

Figura 14 – Comparação das EADs por inalação derivadas de dados *in vitro* cHTS com a concentração estimada no ar baseada no biomonitoramento humano (EDI)



Fonte: Do Autor.

Nota: EAD: *equivalent administered dose*. EDI: *Estimated Daily Intake*. SEEM3: *Systematic Empirical Evaluation of Models*.

Os dados cHTS dos ensaios ativos *in vitro* revelaram bioatividades predominantemente relacionadas ao metabolismo de hormônios esteroides, processos celulares e vias enzimáticas metabólicas impactadas pela exposição ao triazol. Notavelmente, foram identificados distúrbios na biossíntese de androstenediona, testosterona, cortisol, estradiol, estrona, 17-alfa-hidroxiprogesterona e 17-alfa-hidroxipregnenolona na linha celular H295R com 48 horas de exposição química. Além disso, foram observadas bioatividades relacionadas às enzimas CYP450, incluindo CYP19A1 (aromatase), CYP2A2, CYP2C11, CYP2C13 e CYP2C19, em concentrações ambientalmente relevantes. Essas observações resultaram da sobreposição entre os EADs calculados para o

epoxiconazol, determinado usando o modelo oral da ferramenta IVIVE solve_pbtk no ICE. E estes resultados são suportados pelas alterações dos hormônios esteroides, alterações celulares e estresse oxidativo dos biomarcadores humanos avaliados neste estudo. Além disso, para o triadimenol, os dados de exposição humana baseados na EDI excederam um EAD relacionado à modulação da atividade do transportador de dopamina relacionada ao gene SLC6A3.

A ferramenta *Curve Surfer* resultou um número substancial de ensaios ativos *in vitro* tanto para os triazóis quanto para os fungicidas estrobilurina azoxistrobina e piraclostrobina. A inclusão destes ingredientes ativos adicionais revela ainda os diversos alvos moleculares afetados por esta exposição ocupacional a misturas complexas. As estrobilurinas atuam na cadeia respiratória mitocondrial dos fungos, interrompendo o ciclo de ATP e causando estresse oxidativo. Estes fungicidas têm como alvo o complexo III na cadeia de transporte de elétrons mitocondrial, ligando-se ao citocromo b para impedir a transferência de elétrons e a produção de ATP (Kovačević, Hackenberger e Hackenberger, 2021). Embora esta ligação seja reversível, a presença deste sistema em vários organismos levanta preocupações sobre a toxicidade da estrobilurina em espécies não-alvo, incluindo humanos e organismos aquáticos. Além disso, a exposição à estrobilurina está associada ao estresse oxidativo, à apoptose celular, à desregulação endócrina, à cardiotoxicidade, à neurotoxicidade e à genotoxicidade, destacando mecanismos de toxicidade significativos (Leite *et al.*, 2024).

Neste contexto, os dados cHTS confirmam estes mecanismos de ação, mostrando bioatividades predominantes em processos celulares que incluem *mechanistic targets* no ciclo celular, morfologia, proliferação, viabilidade e degradação da matriz extracelular. Embora a parte de biomonitoramento deste estudo não inclua a análise da estrobilurina, os resultados do biomarcadores avaliados mostraram alterações nos marcadores de estresse oxidativo, ácidos biliares plasmáticos e genotoxicidade celular nos voluntários rurais (Costa *et al.*, 2023; Marciano *et al.*, 2024). Além disso, os resultados de IVIVE indicaram que os EADs mais baixos estão relacionados a *mechanistic targets* de processos celulares, respostas ao estresse e metabolismo de hormônios esteroides para esses dois fungicidas. Significativamente, a composição do produto OPERA®, o fungicida mais utilizado na região, lista a piraclostrobina na concentração de 133 g/L (13,3% m/v) e o epoxiconazol na concentração de 50 g/L (5,0% m/v) (BASF, 2023).

Ao analisar a frequência da exposição dos agricultores aos agrotóxicos, o estudo revela um espectro de durações de exposição (Figura 6, seção 5.2.1). Em média, os agricultores estão expostos a atividades de aplicação de agrotóxicos durante 6 meses por ano. No entanto, a proporção dos dados indica que os agricultores trabalham e aplicam agrotóxicos apenas durante alguns meses de cada ano ou durante todo o ano. Esta variabilidade na duração da exposição reflete as diversas práticas agrícolas, tamanhos de propriedade e ciclos de colheita que necessitam do uso de agrotóxicos. Além disso, os dados sobre dias por mês designados para aplicação revelam uma média de 4 dias, sugerindo que os esforços de aplicação estão concentrados em períodos mais curtos e mais intensos. Para as horas diárias despendidas na aplicação de agrotóxicos, foi relatada uma média de 6,9 horas, destacando a parte substancial da jornada de trabalho que os agricultores dedicam a esta atividade. Assim, o valor médio de 7 horas por dia e 4 dias aplicando fungicidas foi utilizado na ferramenta IVIVE para as simulações do modelo de gás PTBK. Estas condições de exposição resultaram na sobreposição de EADs extrapolados nos quais seria esperada bioatividade (com base nos dados cHTS *in vitro*) e as exposições observadas a partir dos dados de biomonitoramento.

Existem informações limitadas sobre os efeitos dos agrotóxicos na saúde feminina em contextos de exposição ocupacional e não ocupacional (Dahiri *et al.*, 2021). Os resultados do biomonitoramento dos triazóis urinários indicam uma exposição significativa entre as mulheres rurais, com resultados para este grupo com os EADs para o epoxiconazol, onde foram observadas bioatividades *in vitro* substanciais.

O modelo inalatório foi empregado para simular condições de exposição ocupacional com base nos dados do questionário referente à frequência de aplicação dos fungicidas. Para o epoxiconazol, a sobreposição com os dados de biomonitoramento e os EADs não foi tão pronunciada como observada no modelo oral, mas houve uma sobreposição com os EADs relacionados à modulação da atividade de enzimas CYP450. A análise também mostra uma pequena lacuna entre a dose diária de inalação e os *mechanistic targets* do metabolismo dos hormônios esteroides. A pequena margem entre os dados humanos e os EADs, utilizando o modelo de gás, também indica uma interação entre os níveis de exposição e estas vias metabólicas específicas, reforçando a relevância da IVIVE para avaliar as vias de exposição por inalação em ambientes ocupacionais (Breen *et al.*, 2021).

O emprego de modelos humanos de PBTK para estimar a exposição crônica em populações potencialmente em risco pode oferecer um reflexo mais preciso do risco humano do que os estudos tradicionais de exposição crônica em animais. Porém, o processo de utilização da EDI para estimar o C ar, para ter unidade em µM/dose, para comparação com os EADs representa uma limitação deste estudo. Para resolver esta questão, apenas a EDI dos agricultores foi considerado representando um cenário de exposição ocupacional. Com base nos dados do questionário relativos às condições de exposição, foi simulada uma duração média de exposição de 7 horas por dia durante um período de 4 dias. Além disso, uma taxa de inalação de 0,053 m³/h/pc foi aplicada para adultos submetidos a exposições agudas de agrotóxicos, refletindo uma taxa de inalação horária de alta intensidade, para tentar uma avaliação mais precisa dos riscos de exposição por inalação (EFSA *et al.*, 2022). Este método sublinha os desafios de avaliar com precisão a exposição por inalação na ausência de dados exclusivos de biomonitoramento por inalação, uma vez que a EDI é derivada das concentrações de triazóis na urina que representa a exposição total para estes fungicidas, que provém da exposição oral, inalatória e dérmica.

Ferramentas computacionais utilizando dados do Tox21 e ToxCast permitiram uma investigação mecanicista de alvos moleculares afetados pela exposição aos agrotóxicos, a partir de ensaios predominantemente baseados em células humanas, estabelecendo-se como meios eficazes para priorização de avaliação de compostos químicos em cenários relevantes para a saúde humana (Wambaugh e Rager, 2022). Pesquisas utilizando dados HTS e ferramentas computacionais demonstrou confiança nos NAMs (ICCVAM, 2024) e suas aplicações para desregulação endócrina (Judson *et al.*, 2015; Kleinstreuer *et al.*, 2017), toxicidade por inalação (Corley *et al.*, 2021; EPA, 2021), e estruturas de testes alternativas para avaliar o potencial de irritação ocular dos agrotóxicos (Clippinger *et al.*, 2021; EPA, 2015). Além disso, a EFSA também destacou o potencial das ferramentas PTBK, ômicas e *in vivo* para a avaliação dos perigos da exposição combinada a múltiplos produtos químicos (EFSA, 2014).

A utilidade de NAMs na avaliação do riscos é exemplificada neste estudo pela distribuição dos resultados IVIVE, nos quais os triazóis, particularmente o epoxiconazol, demonstram EADs mais baixos em cenários de exposição simulados em comparação com o tiametoxam. Este último, um inseticida neonicotinóide, é ativo em apenas seis dos 823 ensaios *in vitro* e apresenta uma distribuição de EAD mais

elevada em relação aos triazóis. Além disso, sua predição de exposição mais baixa no percentil 95, considerada como distante exposição para população, enfatiza o papel das ferramentas computacionais na priorização de compostos químicos na avaliação do risco (Ring *et al.*, 2019). Essas análises permitem uma triagem rápida para avaliação da exposição aos agrotóxicos, destacando a viabilidade de estabelecer PODs com base em eventos de iniciação molecular, que podem potencialmente usar bioatividades *in vitro* para avaliações de risco e avaliações de nível de triagem (Friedman *et al.*, 2019; Health Canada, 2021).

Em contraste, o POD tradicional para epoxiconazol, derivado de um estudo de carcinogenicidade de 18 meses em camundongos, estabeleceu NOAEL em 0,8 mg/kg-pc/dia (EFSA, 2008a). Este POD, depois de ser dividido por um fator de incerteza de 100 que considera as diferenças interespécies (10 vezes) e interindividuais (10 vezes) nos fatores toxicocinéticos e toxicodinâmicos, resulta em uma IDA de 0,008 mg/kg-bw/dia na Europa (EC, 2023). No Brasil, a autoridade reguladora ANVISA fixou esse valor para o epoxiconazol em 0,003 mg/kg-pc/dia (ANVISA, 2024). O nível aceitável de exposição do operador (AOEL) também é definido em 0,008 mg/kg-pc/dia, derivado de um estudo subcrônico em cães também aplicando um fator de segurança de 100 (EFSA, 2008a). Levando esses fatores em consideração, os cálculos de risco indicaram que os grupos rurais com exposição de alta intensidade aos triazóis têm um quociente de risco superior a uma unidade, sugerindo uma alta probabilidade de efeitos tóxicos (Tabela 13, seção 5.6). Com base nos resultados apresentados, os EADs humanos mais baixos poderiam servir como potenciais POD para uma abordagem mais conservadora no cálculo da margem de segurança para fornecer uma estimativa do risco químico para cenários específicos, como a exposição ocupacional e ambiental aos agrotóxicos (Friedman *et al.*, 2019; Zhang *et al.*, 2018).

A aplicação de NAMs e ferramentas computacionais, em alinhamento com dados de biomonitoramento humano e a determinação de biomarcadores, demonstra congruência entre esses métodos e apoia os 3Rs de substituição, redução ou refinamento nas avaliações de toxicidade e riscos químicos (Aylward e Hays, 2011; ICCVAM, 2024). Para promover a progressão de NGRA, ferramentas computacionais que utilizam métodos baseados na biologia humana podem fornecer confiança no discernimento confiável dos níveis de risco, conforme demonstrado neste estudo com os grupos rurais e urbanos (Dent *et al.*, 2021; Thomas *et al.*, 2019). Assim, as

ferramentas de toxicologia computacional podem identificar de forma eficaz compostos químicos para avaliação do risco, preencher lacunas de dados de toxicidade e fornecer informações de exposição para melhor apoiar decisões regulatórias (Health Canada, 2021; Lynch *et al.*, 2024).

6 CONSIDERAÇÕES FINAIS

Este estudo aprimora o conhecimento existente sobre a caracterização do risco de agrotóxicos por meio do biomonitoramento de triazóis urinários como indicadores de dose interna, biomarcadores de efeito e integração de dados de HTS através ferramentas de toxicologia computacional.

A avaliação da exposição foi realizada pelo biomonitoramento dos triazóis urinários utilizando CG-MS para avaliar os níveis de exposição em grupos expostos ocupacional e ambientalmente, em contraste com um grupo urbano não exposto. Biomarcadores de genotoxicidade e de estresse oxidativo indicam um aumento nas alterações celulares, principalmente morte celular por apoptose e necrose, que podem ser decorrentes de desequilíbrio oxidativo, observado principalmente em agricultores expostos ocupacionalmente aos agrotóxicos. As dosagens de ácidos biliares revelaram um aumento nas concentrações de ácidos glicodesoxicólico, taurocólico e taurodesoxicólico nos voluntários expostos, sugerindo que são potenciais bioindicadores de efeito em comparação com o grupo da área urbana, indicando alterações precoces e possivelmente reversíveis, já que não houve diferença estatística nas enzimas hepáticas AST, ALT e γ-GT.

Foram observados parâmetros bioquímicos dos efeitos sistêmicos da exposição ocupacional aos fungicidas, destacando-se como potenciais desreguladores endócrinos. Níveis significativamente reduzidos de testosterona e androstenediona foram observados no grupo dos agricultores, com uma associação inversa de fraca a moderada entre a testosterona e o colesterol, LDL, VLDL, triglicerídeos e glicose, e uma associação direta fraca com níveis de HDL.

Identificou-se perturbações em alvos moleculares que não estão relacionadas apenas à exposição aos fungicidas triazóis, mas também às estrobilurinas presentes nas formulações de agrotóxicos. Além disso, o presente estudo demonstrou a aplicabilidade de ferramentas computacionais para identificar a relação entre essas perturbações *in vitro* e alterações no metabolismo dos hormônios esteroides, nos processos de estresse celular e nas enzimas CYP450. Esses resultados indicam correlação com as alterações dos biomarcadores de efeito na população estudada nos cenários de exposição simulados.

A avaliação de risco para epoxiconazol indica um risco significativo à saúde tanto para homens quanto para mulheres no grupo exposto, especialmente no pior

cenário, onde foi observado o maior valor de triazol urinário, representando uma maior intensidade de exposição. Notavelmente, os resultados observados entre as mulheres do grupo exposto também são alarmantes, enfatizando a urgência da implementação de medidas públicas.

Este trabalho ressalta o papel crítico das ferramentas computacionais na avaliação da bioatividade *in vitro* e exposição aos agrotóxicos, demonstrando uma associação clara e significativa com dados de biomonitoramento humano e efeitos de biomarcadores. Estas estratégias são essenciais para continuar a aumentar a confiança nos NAMs, auxiliando na priorização estudos de toxicidade química, traduzindo as informações de toxicidade dos dados HTS para cenários de exposição humana relevantes e apoiando atividades regulatórias para contribuir para a prevenção de casos de intoxicação e efeitos prejudiciais à saúde em situações de exposição aos agrotóxicos.

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APÊNDICE A – Questionário de investigação de exposição aos agrotóxicos

(Quando a questão não se aplicar anotar o número 99)

I. DADOS DE IDENTIFICAÇÃO

1. Data:		**2. Nº AMOSTRA:**	
3. Nome do município de residência:	**3.1 ( )**	(1) Zona Rural	(2) Zona urbana
4. Endereço: (Rua, Av. etc.):			**Nº:**
5. Bairro:	**6. Ponto de Referência:**	**7. Telefone:**	
8. Nome do município onde trabalha:	**9. Local de Trabalho:**		

II. DADOS DO VOLUNTÁRIO

10. Nome:		**11. Sexo: ( )**	(1)M	(2)F
12. Gestante: ( )			(1)Sim	(2)Não
13. Data de Nascimento:	**14. Idade:**	**15. Anos de estudo*:**		
16. Tabagismo: ( )	**16.1** ( )Atual	**16.2** ( )Anterior	(1)Sim	(2)Não
17. Etilismo: ( )	**17.1** ( )Atual	**17.2** ( )Anterior	(1)Sim	(2)Não
18. Ingestão de café: ( )			(1)Sim	(2)Não

***** A correspondência é feita de tal modo que cada série concluída com aprovação corresponde a 1 ano de estudo.

III. DADOS OCUPACIONAIS

19. Relação de Trabalho: ( )	(1)Proprietário	(2)Assalariado	(3)Meeiro/Arrendatário	(4)Volante
	(5)Outro:			
20. Função: ( )	(1)Administrativa	(2)Téc. Agrícola/Agrônomo	(3) Aplicador na Pecuária	
	(4)Puxa Mangueira	(5)Aplicador/Preparador de Calda	(6) Outros: (Agricultura Familiar)	
21. Contato com Praguicidas: ( )			(1)Sim	(2)Não
22. Há quanto tempo tem contato com praguicidas (venenos)? anos				
23. Frequência do contato com praguicidas: Quantos meses por ano?	Quantos dias por mês?	Quantas horas por dia?		
23.1. Quais meses?	**23.2. Frequência do preparo?**			
24. Quando foi a última vez que teve contato (em dias) com um praguicida?				
25. Com qual produto teve contato pela última vez?				
26. Como aplica os produtos? ( )	(1)Bomba costal (mochila)	(2)Mangueira	(3)Trator sem cabide	
(4)Trator com cabine fechada	(5)Outros (especificar):			
27. Praguicidas de maior utilização	**27.1** Nome comercial:			
	27.2 Princípio Ativo:			
	27.3 Cultura/Lavoura:			

27.1 Como prepara o produto?	

28. Principal Via de Exposição:( )	(1)Cutânea	(2)Digestiva	(3)Respiratória	(4)Outra:
29. Já ficou doente por causa do veneno?( )				(1)Sim (2)Não
30. Quantas vezes você ficou doente por causa do veneno?( )			(1)Uma única vez	(2)Mais de uma vez
31. Alguma vez teve que ser internado?( )				(1)Sim (2)Não
32. Quantas vezes?( )			(1)Uma única vez	(2)Mais de uma vez
33. Há quanto tempo isto aconteceu?( )	(1)Há menos de 10 anos		(2)Há mais de 10 anos	
34. Tipo de Contato:( )	(1)Direto	(2)Indireto	(3)Sem contato	
35. Utiliza Equipamentos de Proteção Individual: ( )	**35.1** ( ) Roupa impermeável apropriada			
35.2 ( ) Bota apropriada	**35.3** ( ) Luvas		**35.4** ( ) Máscaras	
35.5 ( ) Óculos de proteção	**35.6** ( ) Protetor auricular	(1) Completo	(2) Incompleto	(3) Não

IV. DADOS CLÍNICOS

36. Apresenta Doença Cardiovascular: ( )	**36.1**( )Hipertensão arterial (pressão alta)		
36.2 ( )Hipotensão arterial (Pressão baixa)	**36.3** ( ) Arritmia (batedeira)	(1)Sim	(2)Não
37. Apresenta algum sinal/sintoma referente ao Sistema Nervoso Central Periférico? ( )			
37.1 ( ) Dor de cabeça	**37.2** ( ) Fraqueza muscular	**37.3** ( )Tremedeira	
37.4 ( ) Tremor muscular?....Palpebral?	**37.5** ( ) Visão Turva/Vista embaçada		
37.6 ( )Agitação/Irritabilidade	**37.7** ( )Vertigens/Tonturas	**37.8** ( )Formigamento	
37.9 ( )Incoordenação Motora		(1)Sim	(2)Não
38. Do Aparelho Digestório? ( )	**38.1** ( ) Cólicas/Dor de barriga	**38.2** ( ) Dor de estômago	
38.3( )Azia/Queimação	**38.4**( )Náuseas/Enjoo	**38.5**( )Vômito	**38.6**( ) Diarreia (1)Sim (2)Não
39. Do Aparelho Respiratório? ( )	**39.1**( ) Falta de ar	**39.2** ( ) Irritação Nasal (coceira/ardência)	
39.3( ) Catarro ou escarro	**39.4**( )Tosse	(1)Sim	(2)Não
40. Do Aparelho Auditivo: ( )	**40.1** ( ) Diminuição da audição	**40.2** ( ) Zumbido	(1)Sim (2)Não
41. De Pele e Mucosa? O Sr (a) tem alguma coceira relacionada ao uso do agrotóxico? ( )			
41.1 ( ) A coceira veio depois de algum tempo que o sr(a) começou a trabalhar com o produto?			
41.2 ( ) Ou ela aparece logo que usa/prepara o produto? (Irritativa)			
41.3 ( ) O Sr (a) tem irritação ocular (coceira, vermelhidão...), por causa do produto?		(1)Sim	(2)Não
42. Do Aparelho Urinário: ( )	**42.1** ( ) Diminuição da urina (pouco)	**42.2** ( ) Urina escura/com sangue	
42.3 ( ) Outro:		(1)Sim	(2)Não
43. Exposição Raio X ( )	**43.1** Data da última exposição:	(1)Sim	(2)Não

V. NEOPLASIA

44. Tem/Teve Câncer? ( )	**44.1 Qual Tipo?**	
45. Alguém da Família tem/teve Câncer? ( )	**45.1 Qual Tipo?**	
45.2 É da Região? ( )	(1)Sim	(2)Não

VIII. AVALIAÇÃO NUTRICIONAL

52. Nos últimos 7 dias, em quantos dias você comeu os seguintes alimentos ou bebidas?								
ALIMENTO/BEBIDA	**Não comi**	**1 dia**	**2 dias**	**3 dias**	**4 dias**	**5 dias**	**6 dias**	**Todos os 7**
1. Salada crua (alface, tomate, cenoura, pepino, repolho, etc.).								

2. Legumes e verdura cozidos (couve, abóbora, chuchu, brócolis, espinafre, etc.)								
3. Frutas frescas ou salada de frutas								
4. Feijão								
5. Leite ou iogurte								
6. Batata frita, batata de pacote e salgados fritos (coxinha, quibe, pastel etc.)								
7. Hambúrguer e embutidos (salsicha, mortadela, salame, presunto, linguiça etc.)								
8. Bolachas/biscoitos salgados ou salgadinhos								
9. Bolachas/biscoitos doces ou recheados, doces, balas e chocolates (em barra ou bombom)								
10. Refrigerante (não considerar os diets ou light)								

IX. DADOS ANTROPOMÉTRICOS

Peso (Kg)	
Altura (m)	
53. Circunferência abdominal (cm)	

X. CONDUTA

54. Você (ou sua esposa) teve ou tem dificuldade engravidar? ( )	(1)Sim	(2)Não
55. Você (ou sua esposa) sofreu algum abortamento espontâneo? ( )	(1)Sim	(2)Não
56. Tem filhos? ( )	(1)Sim	(2)Não
57. Você tem algum filho com mal formação (congênita)? ( )	(1)Sim	(2)Não
58. Você toma algum medicamento de uso contínuo? ( ) **Qual:**	(1)Sim	(2)Não
59. Nos últimos dois meses você utilizou algum remédio para tratar micose? **Qual:**	(1)Sim	(2)Não

XI - PERGUNTAS FINAIS

60. Apresenta sangramento gengival? ( )	(1) Sim (2) Não	**60.1. Frequência:** ( ) Diária ( ) Semanal ( ) Mensal
61. Apresenta dor nos ossos? ( )	(1)Sim (2) Não	**61.1. Frequência:** ( ) Diária ( ) Semanal ( ) Mensal
62. Teve COVID-19? ( )	(1) Sim (2) Não	**62.1. Gravidade:** ( ) Sem gravidade ( ) Internação Enfermaria ( ) Internação UTI **62.2** Intubação ( ) (1) Sim (2) Não Quanto tempo: _____

63. Apresenta alguma comorbidade relacionada ao aumento da gravidade do COVID?	( ) Hipertensão ( ) Diabetes Mellitus ( ) Obesidade ( ) Doença Pulmonar ( ) Anemia ( ) Alterações de coagulação ( ) Câncer ( ) Imunodeprimido	**63.1. Tomou medicamento:** ( ) (1)Sim (2) Não Quais? _____ **63.2. Apresenta algum tipo de sequela?** ( ) (1) Sim (2) Não Quais?_____
Observações:		

APÊNDICE B – Termo de Consentimento Livre e Esclarecido

Você está sendo convidado a participar, como voluntário, da pesquisa **AVALIAÇÃO DO RISCO DE TRABALHADORES RURAIS EXPOSTOS AOS FUNGICIDAS TRIAZÓIS NO SUL DE MINAS GERAIS**, no caso de você concordar em participar, favor assinar ao final do documento.

Sua participação não é obrigatória, e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento. Sua recusa não trará nenhum prejuízo em sua relação com o pesquisador ou com a instituição.

Você receberá uma cópia deste termo onde consta o telefone, e-mail e endereço do pesquisador principal e participantes, podendo tirar dúvidas do projeto e de sua participação a qualquer momento.

TÍTULO DA PESQUISA: AVALIAÇÃO DO RISCO DE TRABALHADORES RURAIS EXPOSTOS AOS FUNGICIDAS TRIAZÓIS NO SUL DE MINAS GERAIS

PESQUISADORES RESPONSÁVEIS: Luiz Paulo de Aguiar Marciano e Luiz Filipe Costa

ENDEREÇO: Unifal-MG - Rua Gabriel Monteiro da Silva, 700 - Centro, Alfenas.

PESQUISADORES PARTICIPANTES: Profª Dra. Isarita Martins e Profª Dra. Alessandra Cristina Silvério

OBJETIVOS: O objetivo do presente estudo é realizar a avaliação do risco da exposição aos fungicidas triazóis em trabalhadores rurais do sul de Minas Gerais.

JUSTIFICATIVA: Tendo conhecimento dos danos à saúde que os trabalhadores rurais podem apresentar em decorrência da exposição aos fungicidas triazóis, este trabalho propõe uma avaliação do risco destes trabalhadores, devido à escassez de trabalhos na área.

PROCEDIMENTOS DO ESTUDO: No dia da coleta, você responderá um questionário que tem por objetivo conhecer um pouco do seu estado de saúde e manuseio dos praguicidas (para os voluntários da zona rural), de seus hábitos alimentares e uso de medicamentos que possam interferir nos exames. Será coletado uma amostra de urina, em torno de 50-100 mL, três tubos de sangue em um tubo e um raspado com cotonetes de sua boca. Todas as determinações serão realizadas, gratuitamente, nas suas amostras custeadas pela pesquisa, assim como quaisquer despesas decorrentes dela. Essas amostras serão utilizadas para verificarmos se o voluntário está exposto com os fungicidas triazóis e se essa exposição está causando algum problema de sua saúde (para os voluntários da zona rural).

RISCOS E DESCONFORTOS E MEDIDAS: Poderá sentir uma pequena dor

(suportável) durante a coleta de sangue. Para evitar esse desconforto, somente pessoas capacitadas irão coletar as amostras de sangue. Usaremos somente materiais descartáveis e será realizada a antissepsia correta, para evitar possíveis infecções no local da picada da agulha. Também tem o direito e total liberdade de não responder qualquer pergunta do questionário que julgue violar sua privacidade ou causar qualquer tipo de desconforto. Qualquer voluntário pode negar a doação da amostra se julgar desconfortável ou qualquer outro motivo.

O material e dados obtidos na pesquisa serão utilizados exclusivamente para as finalidades descritas neste projeto.

BENEFÍCIOS: Com o resultado da pesquisa poderemos verificar se os trabalhadores estão expostos aos fungicidas triazóis e realizar uma avaliação do risco e orientá-los de forma a minimizar o risco, diminuindo assim a ocorrência de doenças por conta dessa exposição.

CUSTO/REEMBOLSO PARA O PARTICIPANTE: Os exames, despesas e quaisquer danos tidos pelos voluntários serão totalmente custeados pelo projeto de pesquisa, recebendo assistência integral e imediata, não recebendo nenhuma cobrança com o que será realizado. Também não receberá nenhum pagamento com a sua participação.

CONFIDENCIALIDADE DA PESQUISA: As identidades de todos os voluntários serão mantidas em confidencial. Os resultados do estudo serão publicados sem revelar a identidade, entretanto estarão disponíveis para consulta pela equipe envolvida no projeto e pelo Comitê de Ética.

Assinatura do Pesquisador Responsável: _____

APÊNDICE C – Premiações recebidas

Premiações:

1. *Best Abstract Award* da *Exposure Specialty Section;*
2. *Robert J. Rubin Student Travel Award* da *Mechanisms and Risk Assessment Specialty Sections*;
3. *Graduate Students Research Award 2nd Place* da *Clinical and Translational Toxicology Specialty Section*,

com trabalho entiutlado: *Risk Assessment of Exposure to Triazole Fungicides by Human Biomonitoring and Mechanistic Data*, ***63rd Annual Meeting of the Society of Toxicology***, Estados Unidos, 2024.

Menção honrosa pela apresentação oral do trabalho entiutlado: *Exposure assessment of triazole fungicides through human biomonitoring and computational toxicology in Brazilian farmworkers*, ***I International Workshop On Pharmaceutical Research Of Unifal-MG****, Universidade Federal de Alfenas, 2024.*

Menção honrosa pela apresentação oral do trabalho entiutlado: *Optimization of Mass Spectrometry Parameters for the Determination of Multiresidue Pesticides in Food*, ***III Workshop do Programa de Pós-Graduação em Ciências Farmacêuticas****, Universidade Federal de Alfenas, 2022.*

Menção honrosa pela apresentação oral do trabalho entiutlado: *Micronucleus Test in Oral Mucosa as a Bioindicator of Effect in The Monitoring of Occupational Exposure to Triazoles*, ***II Workshop do Programa de Pós-Graduação em Ciências Farmacêuticas/ IX Semana Nacional do Cérebro****, Universidade Federal de Alfenas, 2020.*

ANEXO A – Parecer consubstanciado do Comitê de Ética em Pesquisa

UNIVERSIDADE FEDERAL DE ALFENAS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DE RISCO DE TRABALHADORES RURAIS EXPOSTOS AOS FUNICIDAS TRIAZÓIS NO SUL DE MINAS GERAIS.

Pesquisador: LUIZ PAULO DE AGUIAR MARCIANO

Área Temática:

Versão: 3

CAAE: 34644620.2.0000.5142

Instituição Proponente: UNIVERSIDADE FEDERAL DE ALFENAS - UNIFAL-MG

Patrocinador Principal: UNIVERSIDADE FEDERAL DE ALFENAS - UNIFAL-MG
FUND COORD DE APERFEICOAMENTO DE PESSOAL DE NIVEL SUP

DADOS DO PARECER

Número do Parecer: 4.355.728

Apresentação do Projeto:

Trata-se de um projeto de doutorado, uma pesquisa avaliativa, de natureza epidemiológica mista. O estudo visa realizar o monitoramento biológico dos trabalhadores rurais, através de bioindicadores capazes de avaliar a exposição aos fungicidas e desenvolver metodologias capazes de avaliar o risco dos trabalhadores rurais do Sul de Minas Grais que formulam e aplicam os pesticidas na agricultura. Para a coleta de dados epidemiológicos e clínicos da população, será aplicado um questionário, e para realizar as análises bioquímicas, hormonais, espermograma e análises toxicológicas do bioindicador, será realizada a coleta de sangue, células do epitélio bucal, esperma e urina dos trabalhadores rurais. O intuito do trabalho é verificar se estes trabalhadores rurais do Sul de Minas Gerais apresentam risco aumentado de efeitos tóxicos decorrentes da exposição agregada aos fungicidas triazóis e outros pesticidas. O projeto não apresenta informações sobre conflito de interesses e financiamento do projeto: custeio.

Objetivo da Pesquisa:

O objetivo geral do presente estudo é realizar a avaliação de risco da exposição aos fungicidas triazóis em trabalhadores das zonas rurais do Sul de Minas Gerais.

a) Aplicação de bioindicador de dose-interna por cromatografia acoplada à espectrometria de massas para detecção e quantificação dos fungicidas triazóis em urina de trabalhadores expostos

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ocupacionalmente;
b) Desenvolver e validar método multiresíduo para análise de pesticidas em alimentos hortifrutigranjeiros por cromatografia acoplada à espectrometria de massas;
c) Avaliar a frequência de micronúcleos em linfócitos de sangue periférico, para verificação das alterações cromossômicas, e correlacionar com a concentração de dos triazóis em urina;
d) Determinar os níveis plasmáticos de testosterona total e androstenediona em trabalhadores expostos aos pesticidas, para avaliar a potencialidade como indicador biológico de efeito;
e) Verificar a capacidade reprodutiva dos homens, avaliando a quantidade e a qualidade dos espermatozoides através do espermograma, relacionando com os distúrbios hormonais;
f) Avaliar as funções hepática e renal, por meio da determinação das enzimas aspartato transaminase (AST), alanina transaminase (ALT), gama-glutamil transferase (-GT) e creatinina nos trabalhadores rurais;
g) Verificar as condições de saúde da população por meio da avaliação clínica e das condições de exposição;
h) Analisar estatisticamente os resultados a fim de estabelecer possíveis associações e correlações entre os dados obtidos no estudo;
i) Realizar a avaliação de risco e estabelecer probabilidades de efeitos tóxicos, para subsidiar ações de vigilância sanitária à luz dos resultados obtidos.
Os objetivos são:
a. claros e bem definidos;
b. coerentes com a propositura geral do projeto;
c. exequíveis

Avaliação dos Riscos e Benefícios:
"RISCOS E DESCONFORTOS E MEDIDAS: Poderá sentir uma pequena dor (suportável) durante a coleta de sangue. Para evitar esse desconforto, somente pessoas capacitadas irão coletar as amostras de sangue. Usaremos somente materiais descartáveis e será realizada a antissepsia correta, para evitar

possíveis infecções no local da picada da agulha. Também tem o direito e total liberdade de não responder qualquer pergunta do questionário que julgue violar sua privacidade ou causar qualquer tipo de desconforto. E a coleta da amostra de esperma será feita pelo próprio voluntário em sua residência, evitando quaisquer tipos de desconforto, em dias agendados para entrega da amostra. Qualquer voluntário pode negar a doação da amostra de esperma se julgar desconfortável ou qualquer outro motivo. O material e dados obtidos na pesquisa serão utilizados exclusivamente para as finalidades descritas neste projeto."

b. Há benefícios oriundos da execução do projeto:
Com o resultado da pesquisa poderemos verificar se os trabalhadores estão expostos aos fungicidas triazóis e realizar uma avaliação de risco e orientá-los de forma a minimizar o risco, diminuindo assim a ocorrência de doenças por conta dessa exposição.

Análise CEP:
Os riscos e benefícios são bem descritos e bem avaliados.

Comentários e Considerações sobre a Pesquisa:
a. Metodologia da pesquisa – adequada ao objetivo do projeto.
Foram realizadas as correções, o local onde poderão ser realizadas coletas das amostras. O pesquisador indica este local será decidido com o auxilio da Secretaria Municipal de Saúde, provavelmente no PFS mais próximo da residência do participante. Relatam os critérios de inclusão e exclusão, nas Informações básicas no Projeto completo.
Em Informações básicas, alguns dados da Metodologia Proposta se encontram no item Metodologia de Análise de Dados como por exemplo:
- dados amostrais como características dos voluntários (sexo, faixa etária), número de voluntários, divisão dos grupos (controle e exposto) dados de inclusão e exclusão;
- Etapas da coleta;

-Não citam os laboratórios (LAFT e LACEM), onde serão realizadas as análises. Cabe ressaltar que a metodologia descrita deve constar em todos os documentos apresentados;
b. Em Informações básicas, no item "Haverá retenção de amostra para armazenamento em banco?", a resposta foi sim, entretanto a justificativa mostra que o armazenamento será apenas para a realização da pesquisa até o momento de se realizar as análises, neste caso não haverá armazenamento em banco.
c. Cronograma de execução da pesquisa – coerente com os objetivos propostos e adequado ao tempo de tramitação do projeto. A pesquisa ocorrerá entre 15/10/2020 15/06/2023. As etapas do trabalho estão detalhadas nos documentos.

Considerações sobre os Termos de apresentação obrigatória:
a. Termo de Consentimento Livre e Esclarecido (TCLE) – presente e adequado
b. Termo de Assentimento (TA) – não se aplica
c. Termo de Assentimento Esclarecido (TAE) – não se aplica
d. Termo de Compromisso para Utilização de Dados e Prontuários (TCUD) – não se aplica
e. Termo de Anuência Institucional (TAI) – presente e adequado.
f. Folha de rosto - presente e adequada.
g. Projeto de pesquisa completo e detalhado - presente e adequado.
h. Documento de Informações Básicas do projeto - presente e adequado.
i. Termo de compromisso para desenvolvimento de protocolos de pesquisa no período da pandemia do coronavírus (covid-19) - presente e adequado.

Recomendações:
Não há.

Conclusões ou Pendências e Lista de Inadequações:
Recomenda-se aprovação.

Considerações Finais a critério do CEP:
O colegiado deste CEP emite parecer ad referendum.

UNIVERSIDADE FEDERAL DE ALFENAS

Continuação do Parecer: 4.355.728

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1552010.pdf	16/09/2020 20:19:20		Aceito
Outros	TAI_LATF.pdf	16/09/2020 20:15:54	LUIZ PAULO DE AGUIAR MARCIANO	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_2020_doutorado_LUIZ_PAULO_Plataforma_Brasil.pdf	16/09/2020 20:15:39	LUIZ PAULO DE AGUIAR MARCIANO	Aceito
Outros	Termo_Compromisso_pandemia.pdf	08/08/2020 21:12:05	LUIZ PAULO DE AGUIAR MARCIANO	Aceito
Outros	TAI_2.pdf	08/08/2020 21:09:33	LUIZ PAULO DE AGUIAR MARCIANO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	08/08/2020 21:06:46	LUIZ PAULO DE AGUIAR MARCIANO	Aceito
Folha de Rosto	Folha_de_rosto_preenchida.pdf	07/07/2020 14:52:51	LUIZ PAULO DE AGUIAR MARCIANO	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ALFENAS, 22 de Outubro de 2020

Assinado por:
Angel Mauricio Castro Gamero
(Coordenador(a))

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ANEXO B – Artigos publicados derivados da tese de doutorado

Artigos publicados:

MARCIANO, L. P. A.; KLEINSTREUER, N.; CHANG, X.; COSTA, L. F.; SILVÉRIO, A. C. P.; MARTINS, I. A novel approach to triazole fungicides risk characterization: Bridging human biomonitoring and computational toxicology. **Science of The Total Environment**, [*S. l.*], v. 953, p. 176003, 2024.

MARCIANO, L. P. A.; COSTA, L. F.; CARDOSO, N. S.; FREIRE, J.; FELTRIM, F.; OLIVEIRA, G. S.; PAULA, F. B. A.; SILVÉRIO, A. C. P.; MARTINS, I. Biomonitoring and risk assessment of human exposure to triazole fungicides. **Regulatory Toxicology and Pharmacology**, [*S. l.*], v. 147, p. 105565, 2024.

NUNES, R. F. N.; MARCIANO, L. P. A.; OLIVEIRA, G. S.; CARDOSO, N. S.; PAULA, F. B. DE A.; SARPA, M.; MARTINS, I. Glyphosate contamination of drinking water and the occurrence of oxidative stress: Exposure assessment to rural Brazilian populations. **Environmental Toxicology and Pharmacology**, [*S. l.*], v. 108, p. 104476, 2024.

COSTA, L. F.; MARCIANO, L. P. A.; FELTRIM, F.; FREIRE, J. O.; SILVA, G. B.; SILVÉRIO, A. C. P.; MARTINS, I. Assessment of cellular damage with cytome assay among environmental/occupational triazole. **Chemico-Biological Interactions**, [*S. l.*], v. 383, p. 110689, 2023.

NOLASCO, D. M.; MENDES, M. P. R.; MARCIANO, L. P. DE A.; COSTA, L. F.; MACEDO, A. N. D.; SAKAKIBARA, I. M.; SILVÉRIO, A. C. P.; PAIVA, M. J. N.; ANDRÉ, L. C. An Exploratory Study of the Metabolite Profiling from Pesticides Exposed Workers. **Metabolites**, [*S. l.*], v. 13, n. 5, p. 596, 2023.

Science of the Total Environment 953 (2024) 176003

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

A novel approach to triazole fungicides risk characterization: Bridging human biomonitoring and computational toxicology

Luiz P.A. Marciano [a], Nicole Kleinstreuer [b], Xiaoqing Chang [c], Luiz F. Costa [a], Alessandra C.P. Silvério [d], Isarita Martins [a,*]

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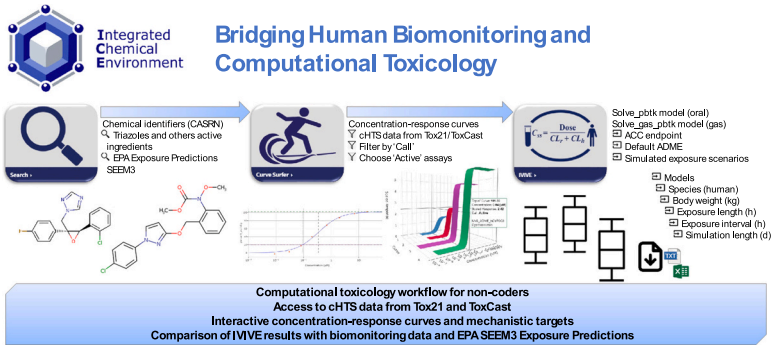
[b] National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA
[c] Inotiv-RTP, Morrisville, NC, USA
[d] University José do Rosário Vellano - UNIFENAS, Alfenas, Minas Gerais, Brazil

HIGHLIGHTS

- High frequency and intensity of triazole exposure was observed in rural areas of Brazil.
- Androstenedione and testosterone levels were significantly reduced in exposed farmworkers.
- *In vitro* human cell-based data and computational modeling predict effects observed in human biomonitoring.
- Analyses support potentially significant human health risk to epoxiconazole exposure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Henner Hollert

Keywords:
Computational toxicology
Exposure assessment
IVIVE
NAM
Biomarkers
Risk assessment

ABSTRACT

Brazil stands as the world's leading coffee producer, where the extensive use of pesticides is economically critical yet poses health and environmental risks due to their non-selective mechanisms of action. Specifically, triazole fungicides are widely used in agriculture to manage fungal diseases and are known to disrupt mammalian CYP450 and liver microsomal enzymes. This research establishes a framework for risk characterization of human exposure to triazole fungicides by internal-dose biomonitoring, biochemical marker measurements, and integration of high-throughput screening (HTS) data *via* computational toxicology workflows from the Integrated Chemical Environment (ICE). Volunteers from the southern region of Minas Gerais, Brazil, were divided into two groups: farmworkers and spouses occupationally and environmentally exposed to pesticides from rural areas ($n = 140$) and individuals from the urban area to serve as a comparison group ($n = 50$). Three triazole fungicides, cyproconazole, epoxiconazole, and triadimenol, were detected in the urine samples of both men and women in the rural group. Androstenedione and testosterone hormones were significantly reduced in the farmworker group (Mann-Whitney test, $p < 0.0001$). The data show a significant inverse association of testosterone with

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cholesterol, LDL, VLDL, triglycerides, and glucose and a direct association with HDL (Spearman's correlation, $p < 0.05$). In the ICE workflow, active *in vitro* HTS assays were identified for the three measured triazoles and three other active ingredients from the pesticide formulations. The curated HTS data confirm bioactivities predominantly related to steroid hormone metabolism, cellular stress processes, and CYP450 enzymes impacted by fungicide exposure at occupationally and environmentally relevant concentrations based on the *in vitro* to *in vivo* extrapolation models. These results characterize the potentially significant human health risk, particularly from the high frequency and intensity of exposure to epoxiconazole. This study showcases the critical role of biomonitoring and utility of computational tools in evaluating pesticide exposure and minimizing the risk.

1. Introduction

Pesticides are chemical substances that act to control insects, fungi, worms, and weeds that affect crops in agriculture. Although there are benefits to their use in agriculture, occupational and environmental exposure poses a threat to public health (Teodoro et al., 2019). Due to their widespread agricultural use, pesticides present significant exposure potential, especially for rural workers who routinely handle these products. Occupational exposure occurs during the production, transportation, preparation, and application of these chemicals in the fields (Ye et al., 2013). However, the pervasiveness of pesticides extends beyond occupational settings, where individuals are also exposed through environmental routes, including household applications, ornamental plant use, laundering soiled garments, contaminated water, food residues, and spray drift (Knapke et al., 2022).

Triazoles fungicides, comprising 21 % of the global fungicide market, are crucial in controlling various fungal diseases in crops like fruits, vegetables, and grains (Cui et al., 2021). The antifungal activity of triazoles stems from their mechanism of action to competitively inhibit CYP51 (lanosterol-14α-demethylase), a key enzyme for the biosynthesis of sterols in fungi. The inhibition of CYP51 causes ergosterol deletion and accumulation of lanosterol and other 14-methylsterols, resulting in changes in fungal cell walls and consequent inhibition of cell growth (Giavini and Menegola, 2010; Tully et al., 2006). These groups of fungicides also exhibit endocrine-disrupting effects, primarily due to their mechanism of action. An important effect of the inhibition caused by triazoles is related to CYP19 (aromatase), which can alter estrogen biosynthesis in humans. This monooxygenase catalyzes the conversion of androgen to estrogen through the cleavage of the methyl group at carbon 10 of the steroid ring of androstenedione and testosterone to produce estrone and estradiol, respectively (Chambers et al., 2014; Jacobsen et al., 2008).

Importantly, in risk assessment of pesticide exposure in humans, key factors include analyzing the chemicals' toxicity, understanding dose-response relationships, and evaluating exposure levels. Effect biomarkers are utilized to assess the impact of the exposure (Damalas and Eleftherohorinos, 2011; Machado and Martins, 2018). Consequently, in the first two stages of risk assessment, researchers and regulators need access to reliable toxicity data. However, the sheer volume of tens of thousands of chemicals needing toxicity evaluation presents a challenge. The practicality of evaluating every new and existing chemical through conventional mammalian acute and chronic toxicity studies is limited by these challenges (Mansouri et al., 2021).

In this context, computational toxicology refers to the use of computational tools to support integrative approaches for toxicological research and chemical safety assessments *via* predictive modeling and complex data analyses for extrapolation and translation between streams of evidence, particularly human biology-based new approach methodologies (NAMs) that serve as alternatives to animal testing (Kleinstreuer et al., 2020; Thomas et al., 2019). Hence, the next generation risk assessment (NGRA) represents a shift towards an exposure-led, hypothesis-driven framework, promising to advance animal-free safety evaluations that are more rapid and potentially more human-relevant (Dent et al., 2021). The U.S. federal Tox21 is an interagency research collaboration that is at the forefront of evolving toxicology practices for the 21st century (Carlson et al., 2022). This collaboration is dedicated to efficiently assessing a variety of substances, including chemicals, pesticides, and medical products, NAMs (Carlson et al., 2022; Thomas et al., 2018).

NAMs are not necessarily newly developed methods. Their innovation lies in their application to regulatory decision-making processes or as substitutes or complements for traditional testing requirements (Zalm et al., 2022). These approaches encompass high-throughput, computational, and human biology-based strategies, playing a crucial role in the development of predictive models. By leveraging high-throughput screening (HTS) data from Tox21 and U.S. Environmental Protection Agency (EPA) ToxCast program, these models can be instrumental in delineating mechanistic bioactivity profiles related to toxicological outcomes (Browne et al., 2018; Krishna et al., 2021).

To further foster the interpretation and application of HTS data and computational toxicology workflows, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) has developed the Integrated Chemical Environment (ICE) (https://ice.ntp.niehs.nih.gov/). The ICE platform is instrumental in enhancing the accessibility and contextual relevance of high-quality curated data, including HTS data (Abedini et al., 2021; Daniel et al., 2022; Hines et al., 2022).

The ICE Curve Surfer tool provides interactive concentration–response curves from HTS *in vitro* assays, retrieved from the EPA's invitrodb version 3.5 (September 2022). The tool supports analysis and extraction of values like the concentration at 50 % of maximal activity (AC50), activity concentration at the activity threshold cutoff (ACC), and top-of-curve. The curated HTS (cHTS) data also integrates chemical quality control details from analytical chemistry results and flags to highlight potentially lower-quality data, and provides enriched biological understanding by linking assays to mechanistic targets and toxicological modes of action.(Abedini et al., 2021; Daniel et al., 2022).

Additionally, the ICE *in vitro* to *in vivo* extrapolation (IVIVE) tool translates *in vitro* activity concentrations into equivalent *in vivo* dose estimates through a process known as "reverse dosimetry" (Fabian et al., 2019). It utilizes physiologically based toxicokinetic (PBTK) models to extrapolate from *in vitro* experimental measurements and predict the *in vivo* exposure dose that would result in equivalent internal concentrations and elicit similar biological responses in animals or humans (Chang et al., 2021). The tool allows the user to choose between AC50 or ACC endpoints from the cHTS data provided by the Curve Surfer tool. It then applies reverse dosimetry modeling, utilizing the EPA's high throughput toxicokinetic (httk) R package, to predict an equivalent administered dose (EAD) under a specific exposure route and dosing scenario to achieve a plasma concentration equal to the selected endpoint. The user-friendly interface, merging the PBTK models with cHTS data, thereby streamlines the modeling process and minimizes the need for extensive technical knowledge for conducting analyses (Abedini et al., 2021; Bell et al., 2018; Hines et al., 2022). In this manner, the predicted bioactive exposure levels can be compared with actual or estimated human exposures to assess potential health risks (Breen et al., 2021; Chang et al., 2022).

Here we have undertaken a multifaceted risk characterization of exposure to triazole fungicides. Human biomonitoring of urinary triazoles through vortex-assisted liquid-liquid microextraction-gas

chromatography coupled to mass spectrometry (VALLME-GC/MS), serves as an indicator of internal dose, coupled with a questionnaire to gather data on exposure conditions and volunteer characteristics. This study also evaluated steroid hormonal disruptions *in vivo* by measuring androstenedione, testosterone, and biochemical markers. We accessed cHTS *in vitro* data to identify sub-cytotoxic biological pathway perturbations and mechanistic targets, and performed IVIVE analysis to compare human biomonitoring data with simulated exposure models for both oral and inhalation routes.

2. Material and methods

2.1. Study population and data collection

Volunteers of the study were from the southern region of Minas Gerais, Brazil, divided into two groups: those occupationally and environmentally exposed to pesticides ($n = 140$) from rural areas, and those not exposed occupationally ($n = 50$) from urban areas to serve as a comparison group in order to ensure the statistical significance of the study. Sampling was carried out for both groups during the period of intensive fungicide use in coffee plantations between December 2021 and March 2022. Eligible volunteers were adults over 18 years old residing and working in rural southern Minas Gerais. The inclusion criteria targeted male coffee farmers directly involved in triazole fungicide application and women residing in rural settings without direct pesticide application exposure. The objective was to recruit as many volunteers as possible from the rural region, allowing broad participation in the biomonitoring aspect of the study. Volunteers with comorbidities that might interfere with and potentially bias the results, such as cancer, were excluded from the study groups.

Each volunteer provided informed consent by signing a consent form. The study was conducted with the approval of the Ethics Committee of the Federal University of Alfenas-MG (CAEE 34644620.2.0000.5141, October 2020), ensuring strict adherence to volunteer privacy and ethical standards. All laboratory analyses performed were offered free of charge, and participants did not receive any financial compensation for their involvement.

During the sample collection phase, a questionnaire was applied to the volunteers to gather data on their pesticide exposure, health status, and demographic background. The questions were orally presented to ensure comprehension, with responses recorded in the questionnaire.

Blood samples were obtained using tubes without anticoagulants (one tube of 8 mL for serum). The samples were processed immediately and subjected to centrifugation at 2000 rpm for 10 min. Following instructions provided by the researchers, volunteers collected urine samples in clean and sterile polypropylene containers, ensuring the volume of each sample was between 50 and 100 mL. All biological samples were collected simultaneously following the completion of the questionnaire. To maintain the integrity of the samples from the point of collection to analysis, they were transported to the laboratory using a thermally insulated box containing Gelox reusable ice packs (TermoGel, São Paulo, Brazil) to ensure a controlled temperature environment. It should also be highlighted that the approach to both collecting and processing samples was uniformly applied across volunteers from both rural and urban areas.

2.2. Triazole fungicides biomonitoring data

The analysis of urinary triazoles was performed by GC–MS (QP 2010 Plus, Shimadzu, Kyoto, Japan) using a VALLME-GC/MS (Machado et al., 2019; Marciano et al., 2024). The method was used to detect the fungicides cyproconazole, epoxiconazole, metconazole, propiconazole, and triadimenol. Reagents and standard specifications, such as purity and manufacturer details, can be found in supplemental material SM1.

For the urine extraction, 100 μL of β-glucuronidase enzyme (diluted 1:28 in 0.5 mol/L acetate buffer, pH 5.0) was added to 1 mL of urine in a falcon tube. The mixture was then incubated at 38 °C for 12 h. After the incubation, 20 μL of the internal standard (Tebuconazole-tert-butyl-d9) was added, along with 2 mL of dibasic sodium phosphate buffer at pH 7.0, 1 mL of acetonitrile, and 200 μL of toluene, serving as the extraction solvent. The mixture was agitated for 1 min and centrifuged at 1650*g* for 5 min. Subsequently, 200 μL of the supernatant was transferred to an eppendorf tube and subjected to drying using a vacuum concentrator/evaporator centrifuge (Centrivap Labconco Corporation, Kansas City, USA) at room temperature for 20 min. The dried extract was reconstituted in 100 μL of toluene, and 2 μL of this solution was injected into the chromatographic system, following the GC–MS conditions described in previous studies (Machado et al., 2019; Marciano et al., 2024). The GC–MS conditions are provided in Supplemental Material SM1. The urinary triazole concentrations were normalized by the urinary creatinine of each sample to account for urine volume and possible variations in element concentrations (Aguera et al., 2022; Marciano et al., 2024).

To interpret the urinary levels of triazole fungicide biomarkers in the context of risk assessment, the Estimated Daily Intake (EDI) for triazoles was calculated (Marciano et al., 2024). This calculation was conducted using the formula (Eq. (1)):

$$EDI = \frac{C.CE}{bw.F} \quad (1)$$

The EDI is in μg/kg-bw/day, C represents the mean concentration of triazole in the volunteers' urine (μg/g crea.), and CE is the reference value for creatinine excretion in urine, derived from adult populations in Brazil (1.22 g crea./day) (Mill et al., 2012). The variable bw stands for the mean body weight of men and women in the exposed group (kg), while F denotes the urinary excretion factor of triazole, with specific values for different compounds detected: 0.27 for cyproconazole (EFSA, 2010), 0.17 for epoxiconazole (EFSA, 2008a), and 0.5 for triadimenol (EFSA, 2008b).

2.3. Biochemical markers and steroid hormones levels in serum

The assessment of biochemical markers was conducted with male ($n = 88$) and female ($n = 52$) volunteers from rural areas, along with male volunteers from urban areas ($n = 25$). These analyses were performed at the Central Clinical Analysis Laboratory of the Federal University of Alfenas, certifying standardized results.

Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) (Friedewald's formula), triglycerides, and glucose levels were measured in serum using automated enzymatic (Trinder) methods.

Hormonal analyses for total testosterone and androstenedione were conducted on serum samples obtained from male volunteers from the rural (n = 88) and urban (n = 25) areas. This analysis aims to investigate the disturbance of male hormones upon exposure to triazoles and their impact due to occupational exposure. Therefore, only male volunteers were selected, excluding rural women volunteers, as the focus is specifically on male hormonal changes. The measurements were conducted at the Central Laboratory of Clinical Analysis of Unifal-MG using two assays: the automated Access Testosterone assay (Beckman Coulter Inc., Fullerton, CA, USA), a competitive binding immunometric assay for testosterone (Dittadi et al., 2018), and the Immulite 2000 assay (Siemens Medical Solutions Inc., Malvern, PA, USA), a chemiluminescent immunoassay (Owen and Roberts, 2007).

The reference values utilized in the LACEN for androstenedione male adults between 18 and 66 years range from 0.6 ng to 3.1 ng/mL. Reference values for total testosterone are 175.0 to 781.0 ng/dL.

2.4. Integrated chemical environment workflow

Initially, the Search tool was used to enter the chemical identifiers (CASRNs) for the detected triazoles. Additionally, other active ingredients from products reported to be used by farmers in the

questionnaire, (azoxystrobin, pyraclostrobin, and thiamethoxam, associated with products PrioriXtra®, OPERA®, and Verdadero® respectively), were also included. Exposure predictions from EPA's Systematic Empirical Evaluation of Models (SEEM3) were accessed for both the detected triazoles and the other active ingredients, and the Search tool results were downloaded in a excel file that contains 5th percentile (P5), median, and 95th percentile (P95) (Ring et al., 2019).

The chemicals were then sent to the Curve Surfer tool, and all cHTS datasets were selected. On the results page, the filter applied was by 'Call', choosing the 'Active' option. All active assays selected for the chemicals were sent to the IVIVE tool. In the input page of the IVIVE tool, the solve_pbtk for oral exposures and solve_gas_pbtk for inhalation exposures were tested. For all analyses, the *in vitro* endpoint chosen was the ACC, and the ADME (chemical Absorption, Distribution, Metabolism, and Excretion) parameter source was set to default (*i.e.* experimental if available, otherwise computationally predicted).

To assess the oral exposure, an interval dose of 10 h was considered to simulate the consumption pattern of individuals eating self-harvested agricultural products twice per day. This consumption pattern is particularly relevant for those residing near coffee plantations and other crops, where pesticide drift potential occurs. In this context, a period of 150 days, from November to March, was selected to correspond with the fungicide application season in the region. These parameters are designed to simulate the probability of rural communities being orally exposed to pesticides daily. Regarding the chosen body weight, the objective was to represent the average body weight of men (75.8 kg) and women (73.1 kg) from the rural group as average between the two (74.45 kg). Because there is no biomonitoring data for the other three active ingredients, the same average value was used to evaluate a broader exposure scenario.

To compare the EADs results from the reverse dosimetry modeling in the IVIVE tool with the human data from the study, it is assumed that the entirety of the EDI originates from oral or inhalation route. This assumption is necessary because the urinary biomarker used for calculating the EDI cannot differentiate among exposure routes. The biological data includes all exposure pathways: oral, inhalation, and dermal, making this assumption essential for the application of the oral and inhalation exposure models. For oral exposure, the EDIs were converted to mg/kg/dose.

Conversely, for the gas model, the biological data from the EDIs were converted into air concentrations (Cair), since the EADs generated by the IVIVE tool are given in μM/dose. Eq. (2) (López et al., 2021) was applied to estimate a daily inhalation dose from measured air concentrations. This procedure is vital for using the urinary triazole data, which indicates total exposure to these fungicides but without the availability of direct measurements of pesticides in the air. Therefore, Eq. (2) simply isolates the air concentration (Cair) (López et al., 2021), allowing the biological data to act as an input parameter for running the gas model in ICE and enabling the comparison of EADs from the IVIVE tool with human data available in the study.

$$Estimated\ Cair = \frac{Daily\ Inhaltion\ Dose.bw}{IR.ET} \quad (2)$$

The daily inhalation dose is considered the same as the EDI (μg/kg-bw/dose) for the male volunteers in the rural group. The estimated concentration in the air (Estimated Cair) is in $\mu g/m^3$, with IR indicating the inhalation rate 0.053 m^3/h/bw (EFSA et al., 2022), bw representing the average body weight of farmworkers (75.8 kg), and ET being the exposure time (7 h). By using the estimated air concentration in $\mu g/m^3$, conversion to μM units is accomplished through the application of the molecular weight of the detected triazoles. The conversions are described in supplemental material, Supplemental Excel File.

To assess the inhalation exposure in the IVIVE tool, the exposure interval was set to 24 h and the exposure length was 7 h (farmers average time applying pesticides per day). The simulation length simulated was 4 days (average number of days actively applying fungicides).

The R code to analyze the cHTS and IVIVE data from ICE can be found in supplemental material, R code and Supplemental Excel File.

2.5. Statistical analysis

The statistical analysis was conducted using R Statistical Software version 4.3.2. The Shapiro-Wilk test assessed data normality. Given the data's non-normal distribution, non-parametric tests were utilized. For multiple comparisons, the Kruskal-Wallis test was employed, followed by Dunn's test for *post hoc* analysis. The Mann-Whitney test was used for pairwise comparisons between groups. Additionally, Spearman's correlation assessed the relationship between hormonal levels and biochemical markers in male volunteers. A significance level of 5 % was established for all analyses.

3. Results

3.1. Exposure assessment

The study included a total of 190 individuals who were categorized into two distinct groups based on their exposure levels to pesticides. The first group comprised 140 rural volunteers who were exposed to pesticides, either occupationally or environmentally, while the second group consisted of 50 urban volunteers, acting as a comparison group. The general characteristics of the study volunteers, grouped by rural and urban areas, are described in Table 1.

The occupational exposure data collected through the questionnaire from the farmers group ($n = 88$) is summarized in Fig. 1.

The most reported triazole fungicides used by workers in the region were primarily three commercial products: OPERA®, PrioriXtra®, and Verdadero®, which contain the following active ingredients: epoxiconazole and pyraclostrobin; cyproconazole and azoxystrobin; and cyproconazole thiamethoxam, respectively.

The VALLME-GC/MS methodology, which includes a vacuum concentrator/evaporator centrifuge drying step, was optimized and validated in line with national and international (ANVISA, 2017, 2012; EMA, 2022; FDA, 2018) guidelines and described in a previous study (Marciano et al., 2024). The method validation details can be found in Supplemental Material SM1.

Table 2 presents the concentration range and EDI of the detected triazoles in the rural group. No samples from the urban group showed signals above the detection limit of the method (2 μg/L for cyproconazole, metconazole, and triadimenol; 5 μg/L for epoxiconazole and propiconazole), confirming the absence of occupational exposure to pesticides in the urban group.

3.2. Biochemical markers and steroid hormones

The distribution and comparison of cholesterol, HDL, LDL, VLDL,

Table 1
Volunteer general characteristics from Southern Minas Gerais, Brazil.

Characteristics	Rural group ($n = 140$)	Urban group ($n = 50$)
Sex n (%)		
Male	88 (63)	25 (50)
Female	52 (37)	25 (50)
Age in years (mean ± SD)	46.0 ± 12.6	25.5 ± 7.1
Body weight in kg (mean ± SD)		
Male	75.8 ± 11.7	75.3 ± 12.8
Female	73.1 ± 15.0	66.8 ± 10.5
Alcohol consumption n (%)	38 (27)	31 (62)
Male n	33	14
Female n	5	17
Smoker n (%)	21 (15)	8 (16)
Male n	13	5
Female n	8	3

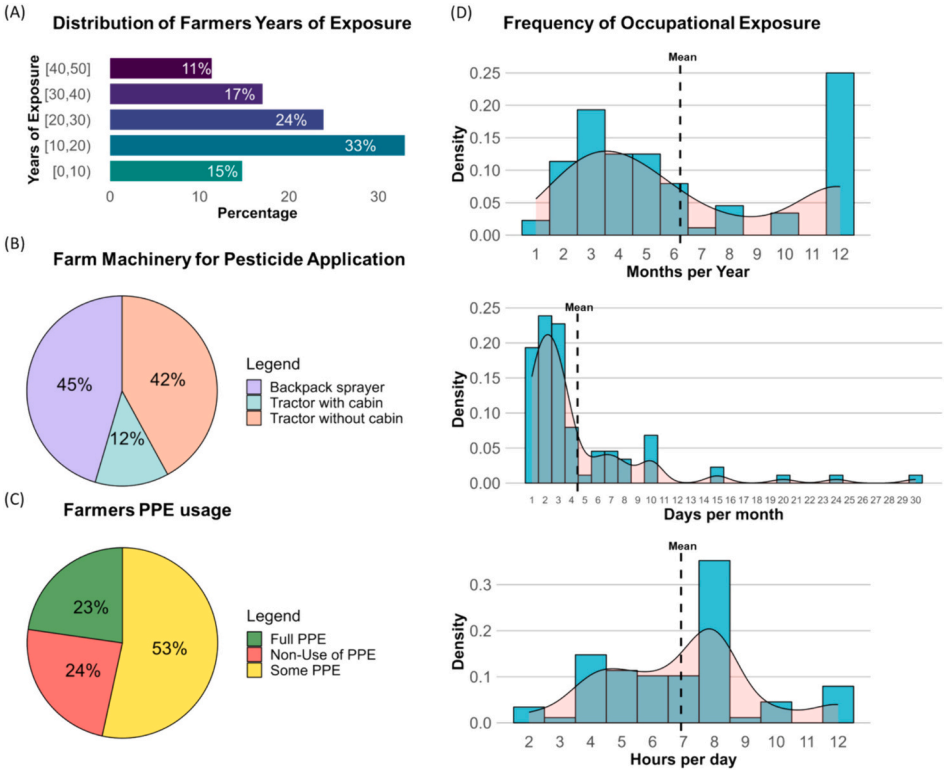

Fig. 1. Farmers occupational exposure conditions (n = 88). (A) Distribution of farmers' years of exposure to pesticides. (B) Farmers self-reported pesticides application machinery. (C) Farmers self-reported personal protective equipment (PPE) usage. (D) Distribution of occupational exposure to pesticides in months per year (mean = 6.2 months), days per month (mean = 4.4 days), and hours per day (6.9 h).

Table 2
Concentration ranges and Estimated Daily Intake (EDI, μg/kg-bw/day) of triazole fungicides detected.

EDI	Range (μg/g creat.)	EDI				
		<LOQ [a]	P25	Median	P75	Max
Farmers						
Cyproconazole	<LOQ–9.6	0.169	0.216	0.235	0.281	0.572
Epoxiconazole	<LOQ–66.7	0.534	0.785	1.08	2.96	6.31
Triadimenol	33.18	–	–	–	–	1.07
Rural women's						
Epoxiconazole	<LOQ–89.3	0.657	1.01	1.89	2.54	8.77

Note: Triazole concentration in urine (μg/g creat.) below the limit of quantification (LOQ); LOQ = 10.0 μg/L for cyproconazole and triadimenol; 30.0 μg/L for epoxiconazole.

triglycerides, and glucose levels through the Kruskal-Wallis test and Dunn's multiple comparison test as a *post hoc* is described in Table 3.

The Kruskal-Wallis test revealed no significant differences among the groups in LDL, VLDL, and triglycerides levels. In contrast, while the non-parametric analysis of variance for cholesterol and HDL levels indicated no significant differences between farmers and urban men, the levels in rural women were significantly different for these markers when compared with the urban men's group (Dunn's test p-values 0.020 and 0.003, respectively). See supplemental material, Table S1 for the summary of all the comparisons. Regarding glucose levels, a significant difference was observed in the farmer group compared to both urban men and rural women (Dunn's test p-values 0.009 and. 0.043, respectively). In the comparison of hormone levels between men in the exposed rural group and those from the urban areas, we observed significant reductions in testosterone and androstenedione levels among the rural volunteers. Fig. 2 illustrates this comparison for both steroid hormones, employing the Mann-Whitney test and the association between the marker's results and steroid hormone levels in male volunteers, we utilized Spearman's correlation test.

The Spearman's correlation analysis revealed a significant relationship between testosterone levels and all evaluated markers (p-value < 0.05). Inverse correlations (rho< 0) were observed, indicating that as testosterone levels decreased, the levels of cholesterol, LDL, VLDL, triglycerides, and glucose tended to increase. Conversely, a direct correlation (rho > 0) with HDL suggested that as testosterone levels increased, HDL levels increased. In contrast, correlations between androstenedione levels and any of the markers were not statistically significant.

3.3. ICE workflow

3.3.1. Search and Curve Surfer tool

The ICE Search tool was used to extract data on the three detected triazoles: epoxiconazole, cyproconazole, and triadimenol, and the three active ingredients, azoxystrobin, pyraclostrobin, and thiamethoxam, from the triazole products, namely OPERA®, PrioriXtra®, and Verdadero®, with reported use. Table S2 presents the pesticides along with the exposure predictions provided by the EPA for these chemicals.

The CASRN of the chemicals were automatically input into the Curve Surfer tool, selecting all available cHTS datasets. The first filter applied was by 'Call', resulting in 755 (16.8 %) assays being classified as active, 3620 (80.4 %) as inactive, and 129 (2.9 %) flag-omit (due to QC or other assay technology interference issues). The 'Active' assays were then chosen for further analysis. Given the large number of active *in vitro* assays and the diversity of mechanistic targets for these pesticides, along with the challenge of graphically representing these results, a new categorization for the mechanistic target groupings of the cHTS data was devised. This categorization was modeled similarly to the existing framework within the Curve Surfer tool in the select assays data input

Table 3
Descriptive statistics and Kruskal-Wallis comparison of the biochemical markers.

Biochemical markers	Group	Mean	SD	Min.	P25	Median	P75	Max	Kruskal-Wallis p-value	Reference values (mg/dL)
Cholesterol[a]	Farmers	181.4	39.5	96.0	163.8	178.5	198.2	351.0	0.046	<190.0
	Rural women's	186.9	37.3	109.0	168.0	180.5	213.5	311.0		
	Urban men's[a]	164.6	33.6	90	144.0	169.0	181.0	239.0		
HDL[a]	Farmers	50.8	11.3	29.0	43	49.0	59.0	89.0	0.008	>40.0
	Rural women's	54.4	10.8	31.0	47.8	54.0	62.5	82.0		
	Urban men's[a]	45.8	11.0	20.0	38	42.0	57.0	63.0		
LDL	Farmers	107.7	35.2	15.8	90.4	107.8	121.3	258.0	0.492	<130.0
	Rural women's	108.1	34.9	25.6	90.1	106.1	127.7	229.8		
	Urban men's	100.6	29.8	47.4	84.6	100.8	111.0	173.6		
VLDL	Farmers	23.0	13.5	6.0	13.4	18.4	29.7	75.2	0.198	<40.0
	Rural women's	24.4	12.5	7.8	15.5	20.4	28.9	56.8		
	Urban men's	18.1	4.6	6.6	15.2	17.0	22.6	26.2		
Triglycerides	Farmers	114.8	67.5	30.0	67.0	92.0	148.5	376.0	0.198	<175.0
	Rural women's	122.1	62.4	39.0	77.8	102.0	144.5	284.0		
	Urban men's	90.7	23.1	33.0	76.0	85.0	113.0	131.0		
Glucose[a]	Farmers[a]	99.4	17.9	75.0	91.8	97.5	102.0	240.0	0.008	<99.0
	Rural women's	98.7	23.0	65.0	86.8	92.5	100.5	194.0		
	Urban men's	92.1	6.2	80.0	87.0	92.0	95.0	111.0		

[a] Significant results based on the Kruskal-Wallis test and Dunn's multiple comparison test as a *post hoc* with 5 % significance.

window.

Table 4 describes the categorization of the mechanist targets from the active assays, and Fig. 3 shows the distribution of mechanistic targets among the active assays, identified using the Curve Surfer tool.

3.3.2. *IVIVE tool*

The IVIVE tool employs PBTK models, specifically the httk package in R, to estimate the EAD based on the activity concentration from the selected active assays through reverse dosimetry. The active *in vitro* assays and the CASRN of the chemicals were automatically input from the Curve Surfer using the 'Send filtered results to' button and selecting the IVIVE tool. To further represent the categorization of the mechanistic targets, the human biomonitoring data, and the SEEM3 exposure predictions, the results of the IVIVE analysis were downloaded into an Excel file from ICE. Following this, the *in vitro* assays were color-coded using R to illustrate the mechanistic targets of EADs and their distribution.

Fig. 4 compares the oral EADs, calculated from the *in vitro* active assays ACC values, the EDIs from the human data, and the SEEM3 exposure predictions. Fig. 5 presents the inhalation EADs, derived using the gas model, in comparison with the EDIs only from farmers, reflecting the available occupational exposure data (from Fig. 1).

4. Discussion

This study developed and applied a comprehensive framework for characterizing the risks associated with pesticide exposure, integrating human exposure data, biomarker measurements, and insights from mechanistic *in vitro* cHTS data using user-friendly computational tools in the ICE.

Within the rural group exposed to pesticides, all 88 farmers actively participated in agricultural practices, notably the application of triazole fungicides, due to the region's emphasis on coffee cultivation (Volsi et al., 2019). The 52 female participants from rural areas also experienced pesticide exposure, both environmentally and through consuming locally grown produce on their family farms. This dual exposure route applies to both the male and female groups within the rural setting (Dereumeaux et al., 2020). Moreover, despite the urban volunteers' younger demographic, each met the study's inclusion criteria, where biomonitoring data confirmed their absence of occupational exposure to pesticides. Previous research in this region has established that urban volunteers with these specific traits serve as a reliable comparison group for such studies (Machado et al., 2021; Silvério et al., 2017).

Regarding occupational exposure, the data indicates that 33 % of farmers have had between 10 and 20 years of exposure, representing a chronic exposure profile among farmers. Notably, 11 % of the farmers have been exposed for the longest duration of 40 to 50 years, and only 15 % for the shortest duration of 0 to 10 years. In terms of pesticide application methods, 45 % of workers reported using a backpack sprayer, 42 % used a tractor without a protective cabin, and 13 % used a tractor with a cabin for pesticide spraying. The use of backpack sprayer for applying fungicides is a significant concern due to the widespread practice among farmers, which significantly increases the risk of inhalation exposure. Importantly, the product leaflet for OPERA®, one of the most commonly used products, explicitly states that this method of application is not permitted in coffee plantations (BASF, 2023).

Full use of personal protective equipment (PPE), which includes pesticide impermeable clothing, waterproof boots, waterproof gloves, a mask, and a protective visor, was reported by only 23 % of farmers, while 53 % reported using at least some equipment and 24 % reported not using any equipment at all. However, in this study, there was no difference observed in the endpoints measured in this study between the complete use of PPE and non or incomplete use, suggesting the potential for misreporting.

The occupational exposure conditions reported in this study are consistent with findings from previous pesticide exposure research in the Southern Minas Gerais region (Machado et al., 2021; Silvério et al., 2017). The reluctance among farmers to consistently use PPE stems from several complex issues, including the discomfort associated with PPE, financial limitations affecting their purchase, and a general lack of education and training, which complicates understanding and adherence to safety protocols (Abreu and Alonzo, 2014; Santana et al., 2016). These elements underscore the necessity for ongoing efforts to educate and equip farmers with the means to protect themselves and reduce pesticide use. Emphasizing the role of risk communication, risk management, and implementing policy measures and programs to reduce occupational pesticide exposure risks.

Triazole fungicides are recognized for their mechanism of action as potent inhibitors of CYP51 (McLean et al., 2006). In humans, CYP51 plays a crucial role in the demethylation process of cholesterol, which is pivotal to producing bile acids, mineralocorticoids, glucocorticoids, and sex steroid hormones (Lepesheva and Waterman, 2011). Given this role, the potential of triazoles to disrupt endocrine functions by interfering with steroid synthesis has been reported (Lv et al., 2017; Machado et al., 2021; Poulsen et al., 2015). To investigate these effects, hormonal analyses were performed on serum samples collected from male volunteers residing in both rural and urban settings. The focus on male volunteers is particularly pertinent to the agricultural context of Southern Minas

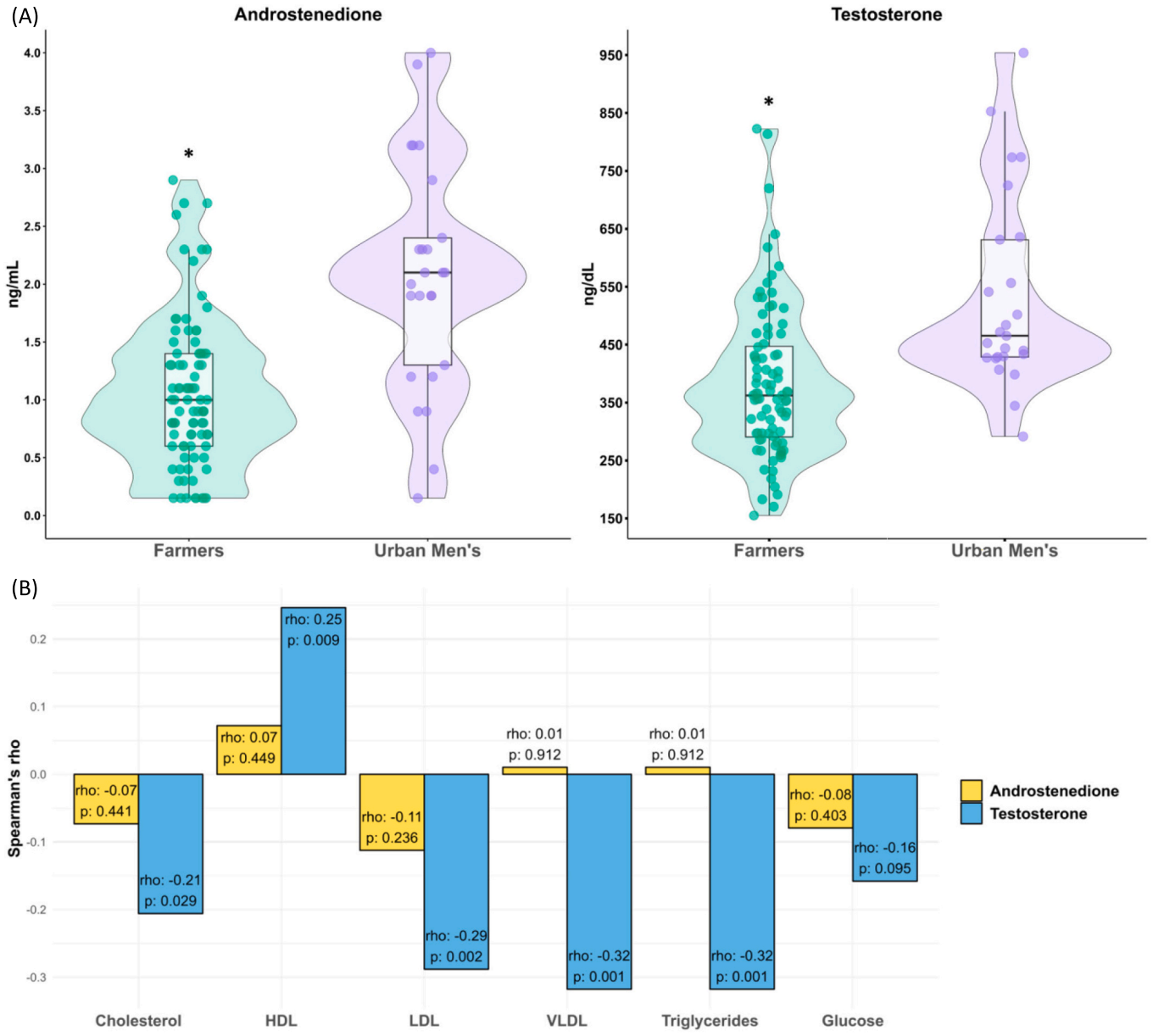

Fig. 2. Steroid hormones comparison and association with biochemical markers (farmers n = 88; Urban Men's $n = 25$). (A) Androstenedione and total testosterone measurements. *p-value obtained using the Mann-Whitney test, with 5 % significance. In the urban men's androstenedione, one outlier point (8.4 ng/mL) was removed. (B) Spearman's correlation of steroid hormones and biochemical markers.

Gerais, Brazil, where pesticide application is predominantly a male activity, thereby aligning with the study's first goal to assess the impact of occupational pesticide exposure on this demographic.

The results indicate that testosterone levels in farmers were significantly lower than those in urban men (Mann-Whitney, p-value < 0.05). However, their median value and 95 % confidence interval remained above the established reference value of 175.0 ng/dL for this hormone. Similarly, androstenedione levels followed a comparable pattern, but with farmer's group measurements approaching the lower reference value of 0.6 ng/mL. Notably, 23 samples from the farmers were equal to or below the reference value for this hormone, contrasting with only 2 samples in the urban group meeting below this threshold.

To further investigate the steroid hormonal alterations, biochemical markers were also measured. Differences in cholesterol and HDL between rural women's group and urban men were observed. Moreover, the notable differences in glucose levels between farmers and the other groups underline the metabolic consequences of prolonged pesticide exposure. Given the established role of glucose metabolism in overall health, these differences may reflect an early indicator of disrupted metabolic homeostasis among the farmer group (Chen et al., 2019).

Spearman's correlation analysis revealed significant associations between testosterone levels and biochemical markers. Reduced levels of testosterone, characterized by increased fat mass, reduced insulin sensitivity, impaired glucose tolerance, elevated triglycerides and cholesterol, and low HDL mirrors metabolic syndrome and type 2 diabetes, both of which contribute to cardiovascular risk (Kelly and Jones, 2013). This pattern underscores the potential influence of occupational and environmental triazole exposure on testosterone levels and its broader metabolic implications.

Expanding upon this, cHTS data from active *in vitro* assays revealed bioactivities predominantly related to the metabolism of steroid hormones, cellular processes, and metabolic enzyme pathways impacted by

Table 4
Categorization of the mechanist targets from the active assays filtered in the Curve Surfer tool (ICE v4.0.2).

Color	Category	Mechanistic targets
	Cellular Process	Cell Cycle Cell Morphology Cellular Proliferation Cell Viability Extracellular Matrix Degradation
	Cellular Stress Response	DNA Damage p53 Modulation Oxidative Stress
	Cytochrome P450 Activity Modulation	Cytochrome P450 Activity Modulation Other Xenobiotic Response Transcription Factors
	Energy Metabolism Process	Energy Metabolism Process Mitochondrial Function
	Epigenetic Process	Histone Modification
	Gene Expression	Aryl Hydrocarbon Receptor Modulation Farnesoid X-activated Receptor Modulation Other Transcription Factors Other Developmental Signaling Transcription Factors RAR-related Orphan Receptor Modulation Retinoic Acid Receptor Modulation Retinoid X Receptor Modulation Vitamin D Modulation
	Immune and Inflammatory Response	Clotting Inflammation
	Malformation	Malformation
	Neuronal Transmission	Adenosine Receptor Modulation Dopamine Transporter Activity Modulation Ion Channel Activity Neurotransmission Opioid Receptor Modulation
	Steroid Hormone Metabolism	Androgen Receptor Modulation Aromatase Activity Modulation Cholesterol Transport Estrogen Biosynthesis and Metabolism Estrogen Receptor Modulation Glucocorticoid Biosynthesis and Metabolism Glucocorticoid Receptor Modulation Progestogen Biosynthesis and Metabolism Progesterone Receptor Modulation
	Thyroid Hormone Metabolism	Thyroid Receptor Modulation Sodium/Iodide Cotransporter
	Vascularization	Vascularization

triazole exposure. Notably, disturbances were identified in the biosynthesis of androstenedione, testosterone, cortisol, estradiol, estrone, 17-alpha-hydroxyprogesterone, and 17-alpha-hydroxypregnenolone in the H295R cell line at 48 h of chemical exposure. Additionally, bioactivities related to CYP450 enzymes, including CYP19A1 (aromatase), CYP2A2, CYP2C11, CYP2C13, and CYP2C19, were observed at environmentally relevant concentrations. These insights stemmed from the overlap between the predicted EADs for epoxiconazole, as determined using the IVIVE tool solve_pbtk oral model in the ICE, and were supported by the biochemical marker alterations. Additionally, for triadimenol, the human exposure data based on EDI exceeded an EAD related to dopamine transporter activity modulation related to the gene SLC6A3.

The Curve Surfer tool shows a substantial number of active *in vitro* assays for both the triazoles and the strobilurin fungicides azoxystrobin and pyraclostrobin. The inclusion of these additional active ingredients further reveals the diverse molecular targets affected by this occupational exposure to complex mixtures. The strobilurins act on the fungal mitochondrial respiratory chain, interrupting the ATP cycle and causing oxidative stress. They target complex III in the mitochondrial electron transport chain, binding to cytochrome *b* to impede electron transfer and ATP production (Kovačević et al., 2021). Although this binding is reversible, the presence of this system in various organisms raises concerns about strobilurin toxicity in non-target species, including humans and aquatic organisms. In addition, strobilurin exposure is linked to oxidative stress, cellular apoptosis, endocrine disruption, cardiotoxicity, neurotoxicity, and genotoxicity, highlighting significant toxicity mechanisms (Leite et al., 2024).

In this context, the cHTS data confirm these mechanisms of action, showing predominant bioactivities in cellular processes that include mechanistic targets in the cell cycle, morphology, proliferation, viability, and extracellular matrix degradation. Although the biomonitoring part of the study did not include the strobilurin analysis, previous studies showed alterations in oxidative stress markers, plasma bile acids, and cellular genotoxicity in the rural volunteers from this study population (Costa et al., 2023; Marciano et al., 2024). Additionally, the IVIVE results indicated that the lower EADs relate to mechanistic targets of cellular processes, stress responses, and steroid hormone metabolism for these two fungicides. Significantly, the OPERA® pesticide product composition, the most used fungicide product in the region, lists pyraclostrobin at a concentration of 133 g/L (13.3 % *w/v*), and epoxiconazole at a concentration of 50 g/L (5.0 % w/v) (BASF, 2023).

In analyzing the frequency of farmers' exposure to pesticides, the study uncovers a spectrum of exposure durations. On average, farmers are exposed to pesticide application activities for 6.2 months each year. However, the proportion of the data indicates that farmers are either working and applying pesticides for just a few months each year or for the entire year. This variability in exposure duration reflects the diverse agricultural practices, property sizes, and crop cycles that necessitate pesticide use. Additionally, the data on days per month spent applying pesticides reveal an average of 4 days, suggesting that application efforts are concentrated in shorter and more intensive periods. For the hours per day spent applying pesticides, an average of 6.9 h was reported, highlighting the substantial part of their workday that farmers dedicate to this activity. The average value of 7 h per day and 4 days applying fungicides was used in the IVIVE tool for the gas PTBK model simulations. These realistic exposure conditions resulted in overlapping extrapolated human exposure values at which bioactivity would be expected (based on *in vitro* cHTS data) and observed exposures from the human biomonitoring data.

Limited information exists regarding the effects of pesticides on female health across both occupational and non-occupational exposure contexts (Dahiri et al., 2021). In this study, the biomonitoring results indicate significant triazole exposure among rural women, with findings for this demographic overlapping with the EADs for epoxiconazole where substantial *in vitro* bioactivities were observed.

The gas model was employed to simulate occupational exposure conditions based on the questionnaire data regarding pesticide application frequency. For epoxiconazole, the overlap with biomonitoring data and the EADs was not as pronounced as seen with the oral model, but there was an overlap with EADs related to CYP450 activity modulation. The analysis also shows a small gap between the daily inhalation dose and steroid hormone metabolism mechanistic targets. The small margin between the human data and the EADs, using the gas model, also indicates an interaction between exposure levels and these specific metabolic pathways, reinforcing the IVIVE relevance for evaluating inhalation exposure routes in occupational settings (Breen et al., 2021).

Employing human PBTK models to estimate chronic exposure in potentially at-risk populations may offer a more accurate reflection of human risk than traditional animal chronic exposure studies. However, the process of using the EDI to estimate the Cair, to have an inhalation μM per dose, for comparison with EAD represents a limitation of this study. To address this, only the EDI from farmers was considered to

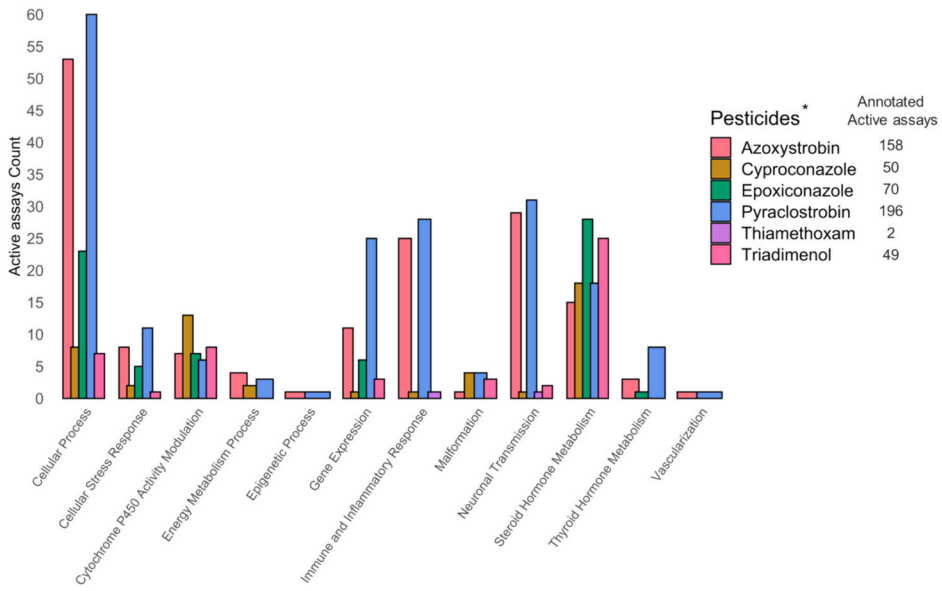

Fig. 3. Mechanistic targets distribution of active assays. *Additional pesticides identified in formulation products used by farmers. Accessed *via* ICE v4.0.2 cHTS data. Filtered out 230 unannotated active assays: Azoxystrobin = 79; Cyproconazole = 31; Epoxiconazole = 0; Pyraclostrobin = 77; Thiamethoxam = 4; Triadimenol = 39.

Fig. 4. Comparison of oral EADs derived from *in vitro* cHTS data, EDI data from human biomonitoring exposure assessment study, and SEEM3 exposure predictions 95th percentiles. EAD: equivalent administered doses. EDI: Estimated Daily Intake. SEEM3: Systematic Empirical Evaluation of Models.

represent an occupational exposure scenario. Based on the questionnaire data regarding exposure conditions, an average exposure duration of 7 h per day over a period of 4 days was simulated. Additionally, an hourly inhalation rate of 0.053 m^3/h/bw was applied for adults experiencing acute exposures to pesticides, reflecting a high-intensity hourly inhalation rate, to attempt an accurate assessment of inhalation exposure risks (EFSA et al., 2022). This method underlines the challenges of accurately evaluating inhalation exposure in the absence of exclusive inhalation biomonitoring data, given that the EDI is derived from the triazole concentrations in urine and represents the total exposure for these fungicides, which comes from oral, inhalation, and dermal exposure routes.

Computational tools utilizing Tox21 and ToxCast data enabled a mechanistic investigation of molecular targets affected by chemical exposure, from predominantly human cell-based assays, establishing themselves as effective means for chemical prioritization in scenarios relevant to human health (Wambaugh and Rager, 2022). Research utilizing HTS data and computational tools has demonstrated confidence in

Fig. 5. Comparison of inhalation EADs with estimated air concentration derived from EDI data from human biomonitoring exposure assessment study. EAD: equivalent administered doses. EDI: Estimated Daily Intake.

NAMs (ICCVAM, 2024) and their applications for endocrine disruption (Judson et al., 2015; Kleinstreuer et al., 2017), inhalation toxicity (Corley et al., 2021; EPA, 2021), and alternative testing frameworks for assessing the eye irritation potential of pesticides and pesticide products (Clippinger et al., 2021; EPA, 2015). Additionally, EFSA also has highlighted the potential of PTBK, omics, and *in silico* tools for the hazard assessment of combined exposure to multiple chemicals (EFSA, 2014). The risk assessment utility of this approach is exemplified here by the distribution of IVIVE results, in which triazoles, particularly epoxiconazole, demonstrate lower EADs in simulated exposure scenarios compared to thiamethoxam. The latter, a neonicotinoid insecticide, is active in only six of 823 *in vitro* assays and displays a higher EAD distribution relative to triazoles. Additionally, its lower P95 exposure prediction, considered far-field exposure, emphasizes its role in study prioritization within risk assessment frameworks (Ring et al., 2019). Such analysis allows rapid screening for pesticide exposure assessment, highlighting the feasibility of establishing points of departure (POD) based on molecular initiating events, which can potentially use *in vitro* bioactivities for safety evaluations and screening level assessments (Friedman et al., 2019; Health Canada, 2021).

In contrast, the traditional POD for epoxiconazole, derived from an 18-month carcinogenicity study in mice, established a NOAEL of 0.8 mg/kg-bw/day (EFSA, 2008a). This POD, after being divided by an uncertainty factor of 100 to account for interspecies (10 fold) and interindividual (10 fold) differences in toxicokinetic and toxicodynamic factors, results in an acceptable daily intake of 0.008 mg/kg-bw/day in Europe. In Brazil, the regulatory authority ANVISA has set this value at 0.003 mg/kg-bw/day (ANVISA, 2024). The acceptable operator exposure level (AOEL) is also set at 0.008 mg/kg-bw/day derived from a subchronic dog study applying a safety factor of 100 (EFSA, 2008a). In this study, the maximum EDI of epoxiconazole for rural male and female volunteers was 0.00631 and 0.00877 mg/kg-bw/day, respectively. Taking these factors into account, a previous risk assessment with the study population indicated that rural groups with high intensity exposure to triazoles have a hazard quotient exceeding one, suggesting a high probability of adverse effects (Marciano et al., 2024). Based on the results presented in this study, the lower human EADs could serve as potential PODs for a more conservative approach in calculating the margin of safety to provide an estimate of chemical risk for specific scenarios such as occupational and environmental pesticide exposure (Friedman et al., 2019; Zhang et al., 2018).

The application of NAMs and computational tools, in alignment with human biomonitoring data and biomarker measurement, demonstrates congruence between these methods and supports the 3Rs of replacement, reduction, or refinement in chemical hazard and risk assessments (Aylward and Hays, 2011; ICCVAM, 2024). To further the NGRA progression, computational frameworks using human biology-based methods can provide confidence in reliably discerning levels of risk, as demonstrated in this study with rural and urban groups (Dent et al., 2021; Thomas et al., 2019). Thus, computational toxicology tools can effectively identify chemicals for risk assessment, fill data gaps in hazard profiles, and provide exposure information to better support regulatory decisions (Health Canada, 2021; Lynch et al., 2024).

5. Conclusions

This study combines human biomonitoring of urinary triazoles with effect biomarkers and computational toxicology workflows to enhance existing knowledge in pesticide risk characterization. Biochemical indicators of endocrine disruption and systemic effects of occupational exposure to pesticides were observed. Significantly reduced levels of testosterone and androstenedione were seen in the farmer's group, where testosterone had a weak to moderate inverse association with cholesterol, LDL, VLDL, triglycerides, and glucose, and a weak direct association with HDL levels. Moreover, this study identifies perturbations in molecular targets related not only to triazole fungicide exposure but also to strobilurin fungicides present in pesticide formulations, and explores the relationship between these perturbations *in vitro* and the biomarker alterations in the study population. This work emphasizes the critical role of *in vitro* and computational tools in evaluating pesticide

bioactivity and exposure, and demonstrates a clear and significant association between bioactivity in human cell-based assays and human biomonitoring and biomarker effects data. These strategies are essential for continuing to build confidence in NAMs, aiding in chemical prioritization, translating toxicity information from HTS data to relevant human exposure scenarios, and supporting regulatory activities.

Abbreviations

AC 50	half-maximal activity concentration
ACC	activity concentration at the activity threshold cutoff
ADME	Absorption, Distribution, Metabolism, and Excretion
AOEL	acceptable operator exposure level
Cair	air concentrations
CASRNs	chemical identifiers
cHTS	curated high-throughput screening
EAD	equivalent administered dose
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EPA	U.S. Environmental Protection Agency
ET	exposure time
GC–MS	gas chromatography coupled to mass spectrometry
HDL	high-density lipoprotein
httk	high throughput toxicokinetic
ICE	Integrated Chemical Environment
ICCVAM	Interagency Coordinating Committee for the Validation of Alternative Methods
IR	inhalation rate
IVIVE	*in vitro* to *in vivo* extrapolation
LACEN	Central Laboratory of Clinical Analysis of Unifal-MG
LDL	low-density lipoprotein
NAM	New Approach Methodologies
NGRA	next generation risk assessment
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NOAEL	no observed adverse effect level
P5	5th percentile
P95	95th percentile
POD	points of departure
PPE	personal protective equipment
PTBK	physiologically based toxicokinetic
SEEM	Systematic Empirical Evaluation of Models
VALLME-GC/MS	vortex-assisted liquid-liquid microextraction-gas chromatography coupled to mass spectrometry
VLDL	very low density lipoprotein

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CRediT authorship contribution statement

Luiz P.A. Marciano: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis. **Nicole Kleinstreuer:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Funding acquisition, Data curation, Conceptualization. **Xiaoqing Chang:** Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis. **Luiz F. Costa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Alessandra C.P. Silvério:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Isarita Martins:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Biomonitoring and risk assessment of human exposure to triazole fungicides

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ABSTRACT

Risk assessment and biomarkers were evaluated in volunteers exposed to triazole fungicides in southern Minas Gerais, Brazil. Volunteers were divided into two groups: occupationally and environmentally exposed to pesticides (n = 140) and those unexposed (n = 50) from urban areas. Urine samples were analyzed by GC-MS for triazoles, and samples from men and women in the exposed group were quantified. Groups were further stratified by sex to evaluate the biomarkers results. Oxidative stress was indicated by biomarker analysis for occupationally exposed men with elevated malondialdehyde levels and reduced superoxide dismutase and catalase activity ($p < 0.0001$). Bile acid levels were also elevated in the exposed group ($p < 0.0001$). Biomarkers in this study suggest recent, reversible changes due to pesticide exposure. Liver enzyme levels showed no significant differences. The highest Estimated Daily Intake for epoxiconazole ranged from 0.534 to 6.31 μg/kg-bw/day for men and 0.657–8.77 μg/kg-bw/day for women in the exposed group. Considering the highest detected urinary triazole value, the calculated Hazard Quotient for epoxiconazole was 0.789 for men and 1.1 for women. Results indicate a health risk associated with environmental triazole exposure, highlighting the importance of biomonitoring in risk assessment to prevent intoxication and assist in mitigating adverse health effects from chronic pesticide exposure.

1. Introduction

The economy in Brazil is based on the agricultural sector since it has favorable climatic characteristics and a large amount of arable land (Souza et al., 2023). Brazil is currently the world's largest coffee producer, and this commodity has played a significant role in the country's history (Silva Souza and Navickiene, 2019). The southern region of the state of Minas Gerais stands out as primary growers of *Coffea arabica*, being responsible for 75.4% of total coffee production (Volsi et al., 2019). However, in order to sustain and maintain its high productivity, the country has relied on the continued use of pesticides, leading to its position as the largest importer of pesticides in the world over the past decade (FAO, 2023; Gonçalves and Delabona, 2022).

Triazoles are the second largest class of fungicides in the world by market value (21%) and are used in agriculture to control a range of fungal diseases such as rust, powdery mildews, and many leaf-spotting fungi on fruits, vegetables, ornamentals, and grain crops (Cui et al., 2021). They are heterocyclic compounds (general molecular formula: $C_2H_3N_3$) and contain a five-membered ring of two carbon atoms and three nitrogen atoms (Souders et al., 2019). The general mechanism of antifungal action of triazoles involves competitive inhibition of CYP51 (lanosterol-14α-demethylase), which is a key enzyme in sterol biosynthesis in fungi. Selective inhibition of CYP51 leads to the depletion of ergosterol and the accumulation of lanosterol and other

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14-methylsterols, causing alterations in the fungal cell wall and subsequent inhibition of fungal cell growth (Giavini and Menegola, 2010; Tully et al., 2006). However, triazoles competitively inhibit CYP51 in fungi, but their action is not selective and also affects humans. Consequently, they inhibit cytochrome P450 and liver microsomal enzymes in mammals, resulting in alterations in biomarkers due to exposure to these compounds (Giavini and Menegola, 2010; Machado et al., 2021).

Risk calculations play a vital role as an important and necessary tool that aims to offer reliable scientific data, ensuring the safeguarding of human health (Carrão et al., 2019). Biomonitoring comprises the assessment of exposure by determining the xenobiotic and its biotransformation products in the body. In this context, biomarkers, obtained by specific measurements that evaluate the interactions between a biological system and the agent, are useful in the assessment of health risks. Biomarkers allow for comparing the levels obtained in the study with reference values, aiding in the evaluation of potential health risks (Hardy et al., 2021; Jakubowski and Trzcinka-Ochocka, 2005; Machado and Martins, 2018). In this context, the measurement of lipid peroxidation products, such as malondialdehyde (MDA), and enzymes superoxide dismutase (SOD) and catalase, serve as effective biomarkers for studying the effects of oxidative stress and the body's antioxidant defense (Simicic et al., 2022). Moreover, lipid peroxidation appears to be one of the molecular mechanisms of pesticide toxicity (Hundekari et al., 2013). As a consequence, these compounds can disrupt biochemical and physiological functions. The measurement of bile acids in blood plasma can serve as a biomarker of early hepatotoxicity effects (Machado et al., 2021; Paiva et al., 2015; Silveira et al., 2022).

In recent studies, researchers have adopted the "internal dose approach" for assessing exposure, which involves estimating intake based on measurements of internal dose (Fernández et al., 2020a; Katsikantami et al., 2019). To this end, biomonitoring was conducted employing an internal dose bioindicator for detecting and quantifying triazoles in urine samples. The aim was to establish a correlation between internal dose measurements and exposure levels. Concurrently, the study evaluated oxidative stress, and assessed early and potentially reversible hepatotoxicity effects by determining plasma bile acids. Based on the data found, the Estimated Daily Intake (EDI) will be calculated, and the risk calculations will be performed to determine Hazard Quotient (HQ), calculated by dividing the EDI with the relevant Acceptable Daily Intake (ADI), to assess the health risk resulting from exposure to triazole fungicides.

2. Material and methods

2.1. Study population design

Sampling was carried out during the intensive use of pesticides in the southern region of Minas Gerais, Brazil, between December 2021 and March 2022. Volunteers with an age above 18 years, who live and work in the rural area of the south of Minas Gerais and who are environmentally and/or occupationally exposed to pesticides were chosen as research participants. As inclusion criteria, male volunteers who work as coffee growers and are responsible for the application of triazole fungicides, as well as women who live in rural areas and have no direct contact with pesticides through application, were invited. We obtained $n = 88$ male volunteers (men's group from the rural area) and $n = 52$ women from the exposed volunteers' families (women's group from the rural area). In addition, samples were collected from a group unexposed to pesticides, residing in the urban area $n = 25$ men, and $n = 25$ women (control group), in order to ensure the statistical significance of the study. The control group comprised individuals who had never been involved in rural work and had no previous occupational exposure to pesticides. It is worth noting that the control group was specifically selected from healthy individuals residing in urban areas. Volunteers who had comorbidities that could interfere and be a possible bias in the results, such as cancer, were not included in the groups.

Furthermore, it is important to note that this study received approval from the Ethics Committee of the Federal University of Alfenas-MG (CAEE 34644620.2.0000.5141). The privacy of each volunteer was strictly respected, and all laboratorial analyses conducted as part of this study were provided completely free of charge. The volunteers received no fees or compensation for their participation.

2.2. Data collection

During the sample collection stage, a questionnaire (SM1 in the supplementary information) was administered through in-person interviews conducted by trained interviewers. The purpose of the questionnaire was to gather data on toxicant handling, exposure conditions, overall health status, symptoms associated with chronic exposure, medication usage, and other factors that could potentially affect the analyses.

Blood samples were obtained through venipuncture using vacuum collection tubes without anticoagulants (one tube of 8 mL for serum) and with EDTA (one tube of 4 mL for plasma). The samples were swiftly processed by researchers and immediately subjected to centrifugation at 2000 rpm for 10 min to separate serum and plasma from other blood components in the respective tubes. Volunteers self-collected urine samples through spontaneous urination in clean and sterile polypropylene containers with a volume ranging from 50 to 100 mL after receiving instructions from the researchers to wash their hands before collection. All samples collected on the days of the sampling were transported to the laboratory by the researchers in a thermal box with reusable artificial ices (Gelox) (TermoGel, São Paulo, Brazil). It is worth noting that there were no differences in data collection or in the handling of biological materials between the rural and urban groups.

2.3. Analysis of triazole fungicides in urine by GC-MS

Urine was analyzed using gas chromatography coupled with mass spectrometry (QP, 2010 Plus, Shimadzu®, Kyoto, Japan) to detect five triazole compounds: Cyproconazole (CIP, purity, 95%) was obtained from Santa Cruz (USA). Epoxiconazole (EPX, purity, 99.2%), metconazole (MET, purity, 99.5%), propiconazole (PRP, mixture of stereoisomers, 99%) from the Pestanal® line, triadimenol (TDN, purity, 98.5%), and Tebuconazole-tert-butyl-d9 (TEB-D9) were obtained from Sigma-Aldrich® (São Paulo, Brazil). *Helix pomatia* β-glucuronidase enzyme (type H-2) was purchased from Sigma-Aldrich (Saint Louis, USA). The HPLC grade methanol used was from Êxodo Científica® (Sumaré, Brazil) and acetonitrile, also HPLC grade, from Dinâmica® Química Contemporânea (Indaiatuba, Brazil). Toluene (pesticide grade) was purchased from Grupo Química® (Penha, Brazil). Dibasic potassium phosphate (purity 98%) and orthophosphoric acid (purity 85%) Vetec® (Duque de Caxias, Brazil) and sodium acetate from Synth® (Diadema, Brazil) were used. Ultrapure water was obtained using a Milli-Q Plus water purification system (Millipore®, Bedford, USA).

The sample preparation followed the method developed by Machado et al. (2019) for vortex-assisted liquid-liquid microextraction, with the modification of adding the drying step of the extracting solvent in the evaporating centrifuge followed by resuspension in toluene. The procedure was optimized and validated using urine from volunteers not exposed to triazoles (blank samples), which was first tested for the absence of triazoles.

The urine extraction process involved 1 mL of urine transferred into a Falcon tube, followed by the addition of 100 μL of the β-glucuronidase enzyme (diluted 1: 28 in 0.5 mol L^{-1} acetate buffer, pH 5.0). The mixture was then incubated at 38 °C for 12 h. Subsequently, 20 μL of the internal standard (TEB-D9) was added, followed by 2 mL of dibasic sodium phosphate buffer at pH 7, 1 mL of acetonitrile, and 200 μL of toluene as the extracting solvent. The sample was thoroughly mixed for 1 min and then centrifuged at 1650 g for 5 min. Next, 200 μL of the supernatant was transferred to an eppendorf tube and subjected to

drying using a vacuum concentrator/evaporator centrifuge (Centrivap® Labconco Corporation, Kansas City, USA) at room temperature for 20 min. The resulting dry extract was resuspended in 100 μL of toluene, and 2 μL of the resuspended extract were injected into the chromatographic system using the same conditions described by Machado et al. (2019) (see SM2 in the supplementary information on chromatographic and spectrometric conditions and method validation). The concentrations of triazole fungicides found in urine were also analyzed and statistically compared after normalization with urinary creatinine.

2.4. Determination of oxidative stress markers

2.4.1. Total protein in human serum

The determination of total proteins was performed using the Bradford method (Zaia et al., 1998). This method utilizes Coomassie brilliant blue dye to measure total serum proteins. A standard solution of bovine serum albumin protein (BSA) from Sigma-Aldrich® (São Paulo, Brazil) was used to generate an analytical curve, 0.2–1.0 mg mL^{-1}, for quantification of the samples. The absorbance was measured at 595 nm using a spectrophotometer (Genesys 10 S Series, Madison, Thermo Scientific). The results of the lipid peroxidation, SOD, and catalase were expressed relative to the protein concentration of each sample.

2.4.2. Lipid peroxidation

Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) in serum, which indirectly reflects MDA production through fluorimetry (Sinnhuber and Yu, 1977). The concentration of MDA was estimated using a standard curve with tetraethoxypropane from Sigma-Aldrich® (São Paulo, Brazil) ranging from 0.3 μmol to 38.4 μmol. For each 150 μL of sample or standard, 750 μL of 1.22 M phosphoric acid (Vetec®, Duque de Caxias, Brazil), 1350 μL of water, and 750 μL of 0.67% TBARS in 50% acetic acid (Dinâmica® Química Contemporânea, Indaiatuba, Brazil) were added. The mixture was homogenized for 30 s, and the samples were then incubated in a water bath at 95 °C for 60 min. After the boiling water bath, the tubes containing the samples were cooled in an ice bath. To each 200 μL aliquot of sample or standard, 1800 μL of methanol and 1000 μL of 1 M NaOH were added. Readings were conducted using a spectrofluorimeter (Cary Eclipse Fluorimeter, Varian, Walnut Creek, USA) with excitation and emission wavelengths set at 532 nm and 553 nm, respectively.

2.4.3. Superoxide dismutase (SOD)

SOD activity was measured in serum samples using the colorimetric assay (Oyanagui, 1984). Serum aliquots were incubated with 0.01 M hydroxylamine, 1 mM hypoxanthine, and 0.0515 U/ml xanthine oxidase from Sigma-Aldrich® (São Paulo, Brazil) at 37 °C for 30 min in the dark. Following the incubation, 1000 μL of a solution containing sulphanilic acid, α-naphthylenediamine, and glacial acetic acid (Dinâmica® Química Contemporânea, Indaiatuba, Brazil) were added and left at room temperature for 20 min. The spectrophotometer was set to 550 nm (UV–Vis Spectrophotometer with Peltier Temperature Control, Madison, Thermo Scientific). The enzyme activity was calculated based on the principle that 1 unit of the enzyme produces 50% inhibition in the reaction. The results were expressed relative to the protein concentration of the sample.

2.4.4. Catalase

Catalase activity was measured using a spectrophotometer in serum samples by monitoring the consumption of H_2O_2 per minute at 240 nm (Aebi, 1984). Sample aliquots were incubated in PBS buffer pH 7.4. The reaction was initiated by adding 10 mM H_2O_2, and the absorbance was continuously recorded for 1 min inside the spectrophotometer (UV–Vis Spectrophotometer with Peltier Temperature Control, Madison, Thermo Scientific). The decomposition kinetics of H_2O_2 were determined using its molar extinction coefficient at 240 nm (43.6 M^{-1} cm^{-1}). The results were expressed in units (U) per milligram (mg) of protein, where one U corresponds to the activity of the enzyme that hydrolyzes 1 μmol of H_2O_2 per minute.

2.5. Quantification of plasma bile acid levels

The determination of bile acids in plasma was performed based on the method developed by Wang et al. (2015) with modifications made by Machado et al. (2021) and Silveira et al. (2022) as described below.

Cholic acid (CA, purity ≥98%), deoxycholic acid (DCA, purity ≥98%), glycodeoxycholic acid (GDCA, purity ≥97%), taurocholic acid (TCA, purity ≥97%), and taurodeoxycholic acid (TDCA, purity ≥97%) were purchased from Sigma-Aldrich® (Steinheim, Germany).

In a 2 mL eppendorf tube, 500 μL of a 75% ethyl alcohol solution was added to 200 μL of plasma. The mixture was vortexed for 1 min and then centrifuged for 10 min at 18,928 g. Next, 350 μL of the supernatant was transferred to another clean eppendorf tube, and 1 mL of ice-cold acetonitrile was added. The solution was vortexed and centrifuged under the same conditions mentioned above. After centrifugation, 600 μL of the supernatant was transferred to another clean eppendorf tube and subjected to drying for 40 min at 80 °C in a vacuum concentrator/evaporator centrifuge (Centrivap® Labconco Corporation, Kansas City, USA). The resulting residue was reconstituted with 100 μL of the mobile phase in a proportion of 90:10 (v/v) (solutions described below) and transferred to an amber glass vial.

The samples were analyzed by UHPLC-MS/MS (LCMS-8030, Shimadzu®, Kyoto, Japan), coupled to a triple-quadrupole type mass analyzer with an ESI interface, operating in negative mode (ESI, −3.5 kV). The chromatographic separation was carried out using the NST 18100 chromatographic column (150 mm × 4.6 mm; 5 μm), preceded by a Supelcoguard LC-18 pre-column (10 mm x 4,6 mm, 5 μm; L x ID, particle size), in an oven with a temperature set at 35 °C. The injection volume was 10 μL. Analysis time of 17.5 min.

The mobile phase consists of methanol/ammonium acetate 5 mmol L^{-1} with 0.012% formic acid (solution A) and ultrapure water/ammonium acetate 5 mmol L^{-1} with 0.012% formic acid (solution B) in a proportion of 90:10 (v/v), with a total flow rate of 0.3 mL min^{-1}.

2.6. Biochemical parameters

The biochemical parameters were assessed exclusively in male volunteers from both the rural and urban (control) groups. This selection was based on the primary focus of the study, which aimed to evaluate occupational pesticide exposure. In the study region of southern Minas Gerais, Brazil, men predominantly serve as pesticide applicators in agricultural settings.

Liver function was evaluated through the measurement of specific enzyme activities, using automated kinetic UV methods, for Aspartate transaminase (AST) (Bergmeyer et al., 1986a), Alanine transaminase (ALT) (Bergmeyer et al., 1986b), and Gamma-glutamyl transferase (γ-GT) (Siekmann et al., 2002). All the analyses were carried out at the Central Clinical Analysis Laboratory (LACEN) of the Federal University of Alfenas, ensuring standardized and reliable results.

2.7. Risk assessment

In order to interpret the urinary levels of triazole fungicide biomarkers in the context of risk assessment, the Estimated Daily Intake (EDI) of triazoles was calculated according to Eq. (1) which was similar to those used in recent studies (Ferreira et al., 2021; Li et al., 2022; Šulc et al., 2022).

$$EDI = \frac{C \,.\, CE}{bw \,.\, F} \quad (1)$$

where EDI: estimated daily intake (μg/kg-bw/day); C: concentration of triazole in urine (μg/g crea.); CE: reference value for creatinine excre-

tion in urine derived from adults in Brazil (1.22 g crea./day) (Mill et al., 2012); bw: mean body weight of men and women in the exposed group (kg); F: urinary excretion factor of triazole (0.17 for epoxiconazole (EFSA, 2008a,b), 0.27 for cyproconazole (EFSA, 2010), and 0.5 for triadimenol (EFSA, 2008b)).

The EDI was divided by the ADI to calculate the HQ (Hazard Quotient), which represents the ratio between the estimated exposure to a substance (EDI) and the levels at which no adverse effects are expected (ADI) (Katsikantami et al., 2019). The HQ allows for assessing the risk of exposure to pesticides. It can be obtained using Eq. (2) having the ADI reference guideline for triazoles. If the HQ is less than one, it is considered to be of low health risk (Fernández et al., 2020a).

$$HQ = \frac{EDI}{ADI} \quad (2)$$

2.8. Statistical analysis

The data were subjected to analysis using the GraphPad Prism program, version 8.0.1 (Dotmatics). For the evaluation of urinary triazole method parameters via GC-MS, the Grubbs test was utilized to identify and exclude outlier data in assessing the linearity of the method. The fundamental assumptions of the linear regression model—normality, independence, and homoscedasticity of variance—were assessed using the Shapiro-Wilk, Box-Pearce, and Breusch-Pagan tests, respectively (see SM2 in the supplementary information).

Statistical comparisons were conducted to assess the oxidative stress, bile acids, and liver enzymes of the study groups. The data are presented as the median with a 95% confidence interval. The normality of the data was assessed using the Shapiro-Wilk test. As the data deviated from a normal distribution, non-parametric tests were employed. The Kruskal-Wallis test was used for evaluating multiple comparisons, followed by Dunn's multiple comparison test as a post hoc analysis. When comparing only two groups, the Mann-Whitney test was applied. The significance level chosen for the study was 5%.

3. Results and discussion

3.1. Study population characteristics

The study population consisted of 190 participants who were divided into two study groups by their exposure to pesticides: rural residents exposed to pesticides (n = 140) and healthy urban adults (n = 50) as a control group. Their characteristics are described in Table 1.

All male volunteers in the exposed group (n = 88) work directly in the fields and reported the application of pesticides, mainly triazole fungicides, since coffee is the predominant crop in the region (Volsi et al., 2019). The women in the exposed group (n = 52) live in rural areas and are also environmentally exposed to pesticides, in addition to consuming food from subsistence family farming. The mean age of rural residents was 46.0 ± 12.6 years for the occupationally and environmentally exposed group, and 25.5 ± 7.1 years for urban residents from the unexposed group. Despite the age of the control group being younger, all participants in this group met the inclusion criteria for this study group: being in good health and not being occupationally exposed to pesticides. In previous studies in the study region itself, the urban zone group with these characteristics proved to be a reliable control group for comparisons (Machado et al., 2021; Silvério et al., 2017).

Regarding the self-reported clinical symptoms during the interviews in the application of the questionnaire, differences in percentages were observed between the groups in terms of alterations in the cardiovascular system, as well as the skin and mucosa. In the exposed group, 30% of participants reported alterations in the cardiovascular system, compared to only 8% in the control group. Additionally, 20% of participants in the exposed group reported alterations in the skin and mucosa, while no such reports were made by the control group. These findings can be attributed to the irritating effects that pesticides have on the skin and dermal tissues toxicity (Damalas and Koutroubas, 2016). However, this adverse effect is not exclusive to triazole fungicides but also to other pesticide classes exposures as well (Silva et al., 2023; Thundiyil et al., 2008).

Table 1
Characteristics of exposed and unexposed groups in Southern Minas Gerais, Brazil.

Characteristics	Exposed (n = 140)	Unexposed (n = 50)
Sex n (%)		
Male	88 (63)	25 (50)
Female	52 (47)	25 (50)
Age in years (mean ± SD)	46.0 ± 12.6	25.5 ± 7.1
Body weight in kg (mean ± SD)		
Male	75.8 ± 11.7	75.3 ± 12.8
Female	73.1 ± 15.0	66.8 ± 10.5
Alcohol consumer n (%)	38 (27)	31 (62)
Smoker n (%)	21 (15)	8 (16)
Education level n (%)		
Primary school incomplete	85 (60.7)	-
Only primary school complete	8 (5.7)	-
Secondary school complete	40 (28.6)	44 (88)
University degree	7 (5.0)	6 (12)
Health Complications Reported n (%)		
Cardiovascular	42 (30)[a]	4 (8)
Nervous system	71 (51)	26 (52)
Digestive	59 (42)	17 (34)
Respiratory	42 (30)	14 (28)
Auditory	30 (21)	4 (8)
Skin and mucous membranes	28 (20)[a]	0
Urinary	18 (13)	3 (6)
Covid-19	8 (6)	20 (40)

[a] p-value was significant and obtained through the Mann-Whitney statistical test, with 5% significance.

3.2. Evaluation of exposure to triazole fungicides

To better evaluate and represent the conditions of exposure to pesticides and occupational and environmental exposure of the volunteers, only the exposed group in the rural area was divided into men and women, are described in Table 2.

Occupational exposure time was stratified, and 70% of volunteers have been working with pesticides for over 10 years. In terms of pesticide application methods, 45% of workers reported using a costal pump, 42% used a tractor without a protective cabin, and 13% used a tractor with a cabin for pesticide spraying. Full use of personal protective equipment (PPE) (pesticide-impermeable clothing, waterproof boots, waterproof gloves, a mask, a protective visor, and ear plugs) was reported to be used by only 23%. While 53% reported using at least some equipment and 24% reported not using any equipment at all. However, in our study, we did not observe a difference in the endpoints measured in this study between the complete use of PPE and non- or incomplete use. The low adherence of farmers to PPE is a complex factor influencing the evaluation of pesticide exposure. This can be attributed to several primary reasons, including the discomfort associated with PPE usage, financial constraints hindering their acquisition, and the correlation with a lower educational level (Santana et al., 2016). The latter factor makes it challenging for individuals to comprehend technical information, thereby characterizing a population that is ill-prepared for handling these substances (Abreu and Alonzo, 2014).

The most commonly reported triazole fungicides used by workers in the region were primarily three commercial products: OPERA®, Prior-iXtra®, and Verdadero®, which contain the following active ingredients: epoxiconazole and pyraclostrobin; cyproconazole and azoxystrobin; and cyproconazole thiamethoxam, respectively.

The internal-dose bioindicator application of urinary triazoles by GC-MS aimed to measure the exposure intensity of the pesticide-exposed group and confirm the absence of exposure in the control group. By

Table 2
Conditions and evaluation of exposure to triazole fungicides of rural resident volunteers.

	Men (n = 88)	Women (n = 52)
Period of pesticide exposure n (%)		
≤10.0 Years	26 (30)	-
11.0–25 Years	35 (40)	-
26.0–40 Years	24 (27)	-
>40.0 Years	3 (3)	-
Mode of application to triazole fungicides n (%)		
Costal pump	40 (45)	-
Tractor without cabin	37 (42)	-
Tractor with cabin	11 (13)	-
Personal Protective Equipment n (%)		
None	21 (24)	-
Incomplete use	47 (53)	-
Complete use	20 (23)	-
Triazole fungicides (commercial product) used n (%)		
OPERA® (epoxiconazole)	58 (66)	-
PrioriXtra® (cyproconazole)	25 (28)	-
Verdadero® (cyproconazole)	4 (5)	-
Range of concentration of triazole fungicides in urine (μg L^{-1})	< LOQ - 70.0	< LOQ - 40.2
Detection range of detected triazoles (μg L^{-1})		
Cyproconazole	< LOQ - 15.0	-
Epoxiconazole	< LOQ - 70.0	< LOQ - 40.2
Triadimenol	45.5	-
Range of concentration of triazole fungicides in urine per urinary creatinine (μg/g)*	< LOQ - 66.7*	< LOQ - 89.3*

LOQ = Limit of quantification. LQ = 10.0 μg L^{-1} for cyproconazole and triadimenol; 30.0 μg L^{-1} for epoxiconazole. *p-value was significant and obtained through the Mann-Whitney statistical test, with 5% significance.

incorporating the drying step in a vacuum concentrator/evaporator centrifuge, the method developed by Machado et al. (2019) was validated and in accordance with national and international guidelines for the analysis of biological samples (see SM2 in the supplementary information). The linear range for cyproconazole, metconazole, and triadimenol was found to be between 10.0 and 200.0 μg L^{-1}, while for epoxiconazole and propiconazole, it ranged from 30.0 to 200.0 μg L^{-1}. The limit of detection (LOD) of the method was 2.0 μg L^{-1} for cyproconazole, metconazole, and triadimenol, and 5.0 μg L^{-1} for epoxiconazole and propiconazole.

As anticipated by observing the data gathered by the questionnaire, the most frequently detected fungicide was epoxiconazole, which was present in 86% of the samples from both men and women residing in rural areas. This finding aligns with the fact that epoxiconazole is the most commonly used triazole fungicide in coffee plantations within the region (Machado et al., 2019). The rural sample concentrations ranged from values below the limit of quantification (LOQ) and higher than the LOD of the method to 70.0 μg L^{-1}. Additionally, cyproconazole was detected and quantified in 12% of the samples, exclusively in male urine samples, ranging from values below the LOQ and higher than the LOD of the method to 15.0 μg L^{-1}. Among these samples, 5% showed simultaneous detection of both epoxiconazole and cyproconazole. Furthermore, one sample exhibited the presence of triadimenol at a concentration of 45.5 μg L^{-1}, despite this pesticide not being reported in the questionnaire. The samples of the volunteers from the urban area were below the LOD of the method, confirming their non-exposure to occupational triazole fungicides.

The relative concentrations of triazole fungicides in urine were analyzed and compared statistically after normalizing them with urinary creatinine levels, evaluating the suitability of this approach by the body burden with the bioindicator of exposure, similar to other biological indicators. The Mann-Whitney test revealed a significant difference ($p = 0.0406$) in the concentration values of epoxiconazole when normalized by urinary creatinine, as shown in Table 2. This analyte was selected due to its higher frequency of detection in the exposed group and its presence in both men and women, providing greater statistical reliability. The normalization with urinary creatinine helps correct for possible variations in element concentrations that may be influenced by urine volume (Aguera et al., 2022).

3.3. Oxidative stress biomarkers

The balance between the production of oxidants, reactive oxygen and nitrogen species, and the body's antioxidant defense plays an important role in maintaining cellular and tissue homeostasis in humans (Simicic et al., 2022). The study conducted by Othmène et al. (2020) evaluated the oxidative effects of triazoles in subchronic exposure to the triazole fungicide tebuconazole in adult male rats, assessing biomarkers of oxidative stress and histopathological changes in cardiac tissue. The results showed that tebuconazole treatment led to increased MDA levels, while SOD and catalase initially increased at doses of 0.9, 9.0, and 27.0 mg/kg body weight but decreased at a dose of 45.0 mg/kg body weight, thereby disrupting the oxidative balance and damaging cardiac tissue (Othmène et al., 2020).

To assess the level of oxidative stress in the studied groups, serum lipid peroxidation was indirectly measured by determining MDA production. SOD and catalase enzymes are part of the antioxidant defense mechanism, working to counteract the excess production of oxidant species. Fig. 1 presents the analysis of variance using the Kruskal-Wallis test for MDA, SOD, and catalase in rural and urban control groups, stratified by sex.

The data from the biomarkers indicate a higher level of oxidative stress in rural men compared to urban men and women in both groups. The group of pesticide applicators exhibited reduced levels of defense biomarkers compared to the urban men (control group), suggesting an imbalance and oxidative stress. Similarly, women from rural areas who were environmentally exposed to pesticides also displayed reduced defense biomarkers compared to urban women (control group). However, they did not exhibit significant MDA production, indicating that the reduction of antioxidant enzyme activities did not affect the oxidative stress. This could explain the lower levels of the defense system.

These results align with previous studies examining occupational pesticide exposure that demonstrated a significant decrease in SOD and catalase activity, respectively, and elevated MDA levels in workers handling organophosphate insecticides (Abbasi-Jorjandi et al., 2020; Surajudeen et al., 2014). However, the study by (Hundekari et al., 2013) which also assessed occupational exposure to organophosphates, showed high MDA levels in the exposed group compared to the control group but observed an increase in the activities of SOD, catalase, and glutathione peroxidase (GPx), suggesting an adaptive response to oxidative damage caused by organophosphate exposure. There is also a link between occupational exposure to organophosphate pesticides and genotoxicity that found a marginally significant decrease in SOD and catalase activities among workers applying these pesticides and observed a positive correlation between DNA damage parameters, measured through the comet assay, a type of genotoxicity biomarker, and MDA levels (Zepeda-Arce et al., 2017).

3.4. Determination of plasma bile acid levels and biochemical parameters

The determination of bile acids as an effect bioindicator is crucial in the quest for a method to identify the risks and reverse potential liver damage caused by triazole fungicides. This is particularly important given the evidence of hepatotoxicity associated with these agents (Ekman et al., 2006; Goetz et al., 2006; Heise et al., 2015; Jardim et al., 2018).

The linear range, accuracy, and precision for bile acids CA, DCA, and GDCA were validated within the range of 10.0–500.0 μg L^{-1}, while for TCA and TDCA, they were validated within the range of 25.0–500.0 μg L^{-1}, using the guidelines of the National Health Surveillance Agency

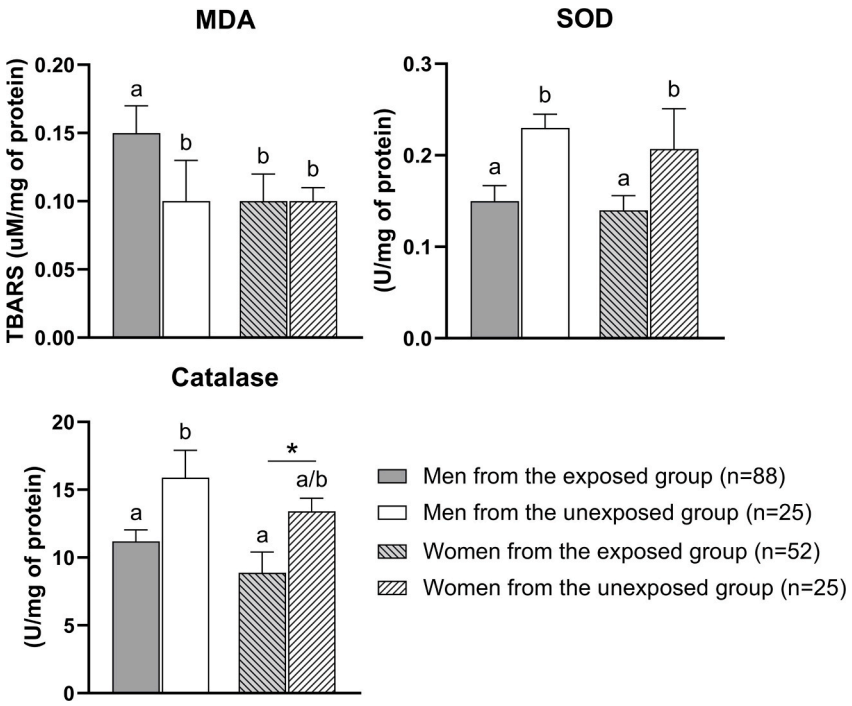

Fig. 1. Determination of oxidative stress biomarkers. Values expressed as median and 95% confidence intervals. Different letters indicate groups that were significantly different ($p < 0.05$) from one another based on Dunn's multiple comparison test as a post hoc, with 5% significance. Groups with the same letters did not show a significant difference from one another based on a post hoc test.

(ANVISA) (BRASIL, 2012) and the Food and Drug Administration (FDA, 2018).

Moreover, Luo et al. (2018) and Sugita et al. (2015) have reported significantly higher concentrations of bile acids in patients with liver failure compared to healthy individuals. Consequently, bile acids have been investigated as potential biomarkers for liver damage. Fig. 2 presents the analysis of variance using the Kruskal-Wallis test for CA, DCA, GDCA, TCA, and TDCA measures in the plasma of rural and urban control groups, stratified by sex.

The data reveal a significant increase in bile acids among men in the exposed group ($p < 0.0001$ from the Kruskal-Wallis test followed by Dunn's test as a post hoc), except for DCA, where no dosage disparity existed between the groups. The CA levels were only significantly elevated in men from the exposed group and exhibited statistical equivalence when comparing women from rural and urban areas. Nevertheless, the biomarkers GDCA, TCA, and TDCA exhibited a statistically significant ($p < 0.0001$) distinction between residents of rural and urban areas (control group).

Studies have consistently shown that bile acids are present at higher plasma concentration levels in patients with liver damage compared to healthy individuals (Bathena et al., 2015; Luo et al., 2018; Trottier et al., 2011, 2012; Woolbright et al., 2014). It is worth noting that in their study, Luo et al. (2018) specifically compared healthy individuals with hospitalized patients who had experienced various cases of liver damage.

Among the bile acids studied, those conjugated with glycine have

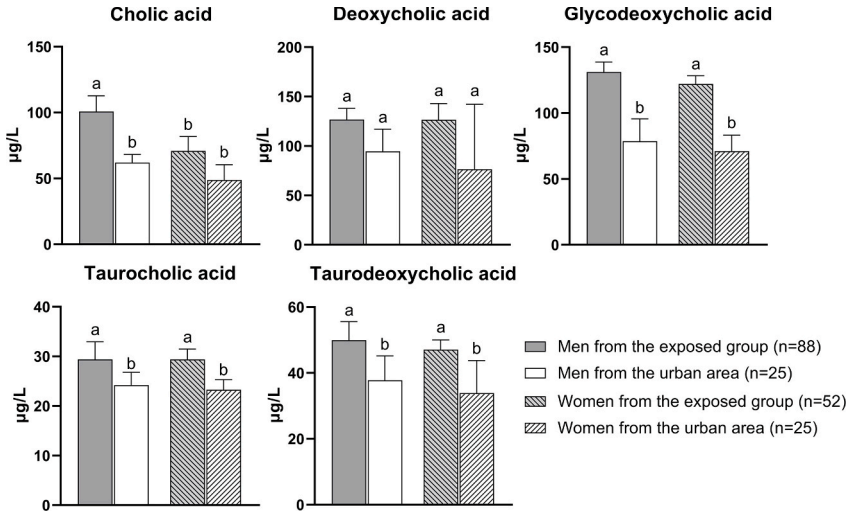

Fig. 2. Determination of plasma bile acid levels. Values expressed as median and 95% confidence intervals. Different letters indicate groups that were significantly different ($p < 0.05$) from one another based on Dunn's multiple comparison test as a post hoc, with 5% significance. Groups with the same letters did not show a significant difference from one another based on a post hoc test.

been found to have the highest concentrations. This can be attributed to the hydrophobic nature of these acids, as conjugation reduces their hydrophobicity (Ashby et al., 2018; Luo et al., 2018; Machado et al., 2021). Unconjugated bile acids, on the other hand, are more hydrophobic and cytotoxic, leading to mitochondrial damage, and cell membrane rupture and potentially causing necrosis and apoptosis in hepatocytes due to the generation of free radicals (Bechmann et al., 2013; Guicciardi et al., 2013; Jang et al., 2012; Luo et al., 2018). Other studies (Goetz and Dix, 2009; Machado et al., 2021) report that transporters involved in steroid metabolism, cholesterol, amino acid, and bile acid absorption were over-regulated by exposure to triazoles, indicating increased uptake of these pesticides by hepatocytes, thereby increasing the damage caused by them and the subsequent excretion of their metabolites. Although diet can influence bile acid concentration to some extent, the impact of dietary factors is minimal compared to the increase associated with liver damage and toxicity (Bathena et al., 2015).

In liver damage, the cell membrane of hepatocytes is disrupted, therefore the enzymes AST, ALT, and γ-GT are released into the bloodstream, causing increased plasma levels and indicating the presence of liver damage (Masubuchi et al., 2016; Woreta and Alqahtani, 2014). As alterations were observed in both biomarkers of oxidative stress and bile acids among men in the exposed group, liver enzymes of male volunteers from both the exposed and unexposed groups were measured (see SM3 in the supplementary information).

The data show that there were no statistically significant difference between the groups through the Mann-Whitney test, as they exhibit very similar values in the measurement of the three biochemical parameters. The reference range for AST is 5.0–34.0 U L^{-1}, while for ALT it is 0–55.0 U L^{-1}. Regarding γ-GT levels, the reference value falls within the range of 12.0–64.0 U L^{-1} for men.

The volunteers exposed to pesticides showed lower alcohol consumption (27%), compared to the unexposed group from the urban area (62%). This differential in alcohol consumption is critical to consider as a potential confounding factor in the biomarker analysis. However, the significant findings related to oxidative stress and increased bile acids seem unaffected by this variable. It's important to note that the control group, which was not exposed to pesticides, had the highest rate of alcohol consumption, underscoring the reliability of these results. In the context of smoking habits, the difference was minimal, with the exposed and unexposed groups showing 15% and 16%, respectively, indicating that this variable does not significantly influence the effect of pesticide exposure on the studied biomarkers. These trends are consistent with others research in the field (Costa et al., 2023; Silvério et al., 2017).

Therefore, it is possible to infer that the studied biomarkers are demonstrating early and possibly reversible alterations, as there have not yet been significant changes in the biochemical tests used for diagnosing liver disease. This hepatotoxic effect of triazoles was also cited by other studies (Ekman et al., 2006; Heise et al., 2015; Machado et al., 2021; Tully et al., 2006).

3.5. Risk assessment

The results of the EDI calculation and the values used are presented in Table 3. EDI was calculated for urinary triazole concentrations (μg/g crea.) ranging from values below the LOQ and higher than the LOD of the method, and the 25th percentile, the 50th percentile (median), the 75th percentile, and the maximum detected. The data demonstrate that the EDI of epoxiconazole is higher compared to other triazoles, primarily due to its elevated concentration in the collected urine samples and its low F value (0.17) (EFSA, 2008a). Furthermore, the ADI for epoxiconazole is lower at 0.008 mg/kg-bw/day (EFSA, 2015), in contrast to the other detected triazoles, which have ADI values of 0.01 mg/kg-bw/day and 0.05 mg/kg-bw/day, according to EU Pesticides Database (EC, 2023) for cyproconazole and triadimenol, respectively. This discrepancy results in higher HQ values when comparing epoxiconazole to cyproconazole and triadimenol, as described in Table 3.

Table 3
Estimated Daily Intake (EDI, μg/kg-bw/day) and Hazard Quotient (HQ) of triazole fungicides detected.

EDI	< LOQ[a]	P25	Median	P75	Max	ADI
Men of exposed group						
Cyproconazole	0.169	0.216	0.235	0.281	0.572	10.0
Epoxiconazole	0.534	0.785	1.08	2.96	6.31	8.0
Triadimenol	-	-	-	-	1.07	50.0
Women of exposed group						
Epoxiconazole	0.657	1.01	1.89	2.54	8.77	8.0
HQ						
Men of exposed group						
Cyproconazole	0.017	0.022	0.024	0.028	0.057	
Epoxiconazole	0.067	0.098	0.134	0.371	0.789	
Triadimenol	-	-	-	-	0.021	
Women of exposed group						
Epoxiconazole	0.082	0.126	0.236	0.317	1.1	

[a] Triazole concentration in urine (μg/g crea.) below the limit of quantification (LOQ); LOQ = 10.0 μg $L^{-1}$for cyproconazole and triadimenol; 30.0 μg $L^{-1}$for epoxiconazole; 25th percentile, 50th percentile (median), 75th percentile and maximum detected value. ADI = Acceptable Daily Intake (μg/kg-bw/day).

In the men of the exposed group, the HQ did not exceed one unit in the maximum detected value of urinary triazole found in the male group, indicating no significant health risk based on the calculations. This data is not supported by the results presented in this study, which reveal potential effects resulting from pesticide exposure, particularly triazole fungicides, as evidenced by biomarkers of oxidative stress and levels of bile acids. These effects are particularly notable in the coffee-growing region, where these fungicides are predominantly used (Machado et al., 2021).

Furthermore, it is worth highlighting the calculated HQ for women in the exposed group, which exceeded one unit (1.1), indicating a high health risk. This observation is attributed to the maximum detected value of urinary triazole found in the female group, originating from a sample with a high concentration of epoxiconazole (40.2 μg L^{-1}). When this sample was normalized by urinary creatinine (0.45 g L^{-1}), this resulted in a significantly higher urinary concentration value (89.3 μg/g crea.), consequently a higher EDI and HQ value. This highlights the significance of considering potential variations in substance concentrations, which can be influenced by urine volume (Aguera et al., 2022).

Regarding the ADI values used, if the ADI indicated by the Brazilian authority (ANVISA, 2023), for epoxiconazole was considered, being 0.003 mg/kg-bw/day the HQ would exceed one for men, for the maximum value of triazole urine detected in both men and women from the exposed group. Thus, in the worst-case scenario, with the greater intensity of exposure represented by the highest urinary triazole value found, male pesticide applicators would have a significant health risk. Thus, the risk calculations performed would not underestimate the occupational risk and would be more representative, considering the exposure conditions, such as the majority of non-or incomplete use of PPE by workers and chronic occupational exposure to triazole fungicides, as reported in Table 2.

Few studies have assessed the risk of triazole fungicides resulting from occupational and environmental exposure. In Czech adults and children, tebuconazole, a triazole fungicide, was detected in nearly all urine samples, indicating exposure primarily driven by diet and suggesting a less significant impact of environmental exposure without a clear association to proximity or agricultural areas surrounding residences, resulting in a lower risk (Šulc et al., 2022). However, our study reveals contrasting findings, as both men and women living in rural areas exhibit higher levels of environmental exposure to fungicides. This distinction is particularly evident in the results observed among women who are not occupationally exposed, and in the worst-case scenario (sample with maximum triazole concertation detected), HQ indicates a higher risk for health.

Recent studies have utilized biological samples to evaluate the potential risk associated with pesticide exposure in populations with specific characteristics. For instance, in Spain, metabolites of organophosphate pesticides, herbicides, and pyrethroids were analyzed in lactating mothers. The findings indicated that factors such as proximity to agricultural activities, place of residence, and the presence of gardens or plants in homes significantly contributed to urinary levels of pesticide metabolites (Fernández et al., 2020a). Additionally, another study conducted in Spain (Fernández et al., 2020b) assessed the risk of organophosphate pesticide, herbicide, and pyrethroid exposure in children, while a study in Portugal focused on glyphosate exposure (Ferreira et al., 2021). While none of these studies provided evidence of health risks, they did not apply potential biomarkers of effect to examine possible recent changes from pesticide exposure, as we did in our study.

4. Conclusions

Therefore, our study contributes new data to the risk assessment of pesticides through biomonitoring, particularly regarding exposure to triazole fungicides among rural residents who experience both occupational and environmental exposure, in addition to relying on their own agricultural crops for sustenance. It was possible to identify and quantify triazole fungicides in the urine of volunteers in the exposed group, demonstrating the applicability of the method as a bioindicator of internal dose and conducting an evaluation of the exposure in the rural area group. The importance of normalizing the results based on urinary creatinine is a crucial step when considering the volume of the collected urine sample. Oxidative stress biomarkers indicate an oxidative imbalance condition in male volunteers occupationally exposed to pesticides. Bile acid measurements revealed an increase in the concentration of glycodeoxycholic, taurocholic, and taurodeoxycholic acids in the volunteers of the exposed group, showing potential bioindicators of effect when comparing the rural area group with the urban area group. This indicates recent and possibly reversible changes, as there was no statistical difference in liver enzymes AST, ALT, and γ-GT, indicating no hepatotoxicity. The risk assessment for all triazoles indicated no significant health risk for men in the exposed group. However, our study reveals effects resulting from exposure to pesticides, particularly triazole fungicides, despite the fact that the risk calculations performed underestimate the probability of adverse effects, except at the highest exposure intensity when considering the ADI value for epoxiconazole in Brazil. Furthermore, the results observed among women in the exposed group are also alarming, especially under the worst-case scenario where the highest urinary triazole value was observed, representing a higher intensity of exposure and highlighting the urgency of implementing public measures.

Thus, this work reinforces the importance of biomonitoring pesticides in risk assessment as a means to contribute to the prevention of intoxication cases and adverse health effects in situations of chronic exposure.

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CRediT authorship contribution statement

Luiz P.A. Marciano: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Luiz F. Costa:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Naiane S. Cardoso:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Josiane Freire:** Investigation. **Fernando Feltrim:** Investigation. **Geovana S. Oliveira:** Investigation. **Fernanda B.A. Paula:** Writing – review & editing, Project administration, Methodology. **Alessandra C.P. Silvério:** Writing – review & editing, Project administration. **Isarita Martins:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2024.105565.

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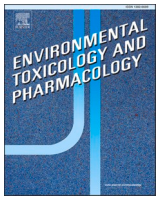

Glyphosate contamination of drinking water and the occurrence of oxidative stress: Exposure assessment to rural Brazilian populations

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ABSTRACT

Studies reported that continuous application of glyphosate can cause disturbance in aquatic/terrestrial environments. As such, the objective of this study is to discuss the risk of exposure to the herbicide in drinking water and to assess the oxidative stress in the consumers rural populations of Casimiro de Abreu/ RJ and Paraguaçu/ MG, Brazil. For this, water samples (n=69) were analysed from the home of volunteers, by FMOC derivatizing-LC-FLD method. The oxidative stress was analysed determining lipid peroxidation (MAD) and defense enzymes (SOD and CAT) in serum samples from rural population (n=42) compared to urban residents (n= 42). Results of the analysis from drinking water, despite the low and moderate risk, by the hazard quotient (HQ), revealed that the population is environmentally exposed to the glyphosate. The relevant findings showed that is important to implement monitoring/ biomonitoring programs to prevent pollution and toxic effects in the rural populations.

1. Introduction

The use of pesticides in agriculture is one of the main causes of contamination in surface and groundwater. These contaminations occur mainly due to the actions of leaching, drainage, and runoff of toxic substances present in crops and soil, which will reach water streams or reservoirs. Contamination of these waters can result in toxic effects for aquatic fauna and flora and for human health due to their use for consumption and leisure practices. Pollution of continental and marine water bodies is predominantly caused by pesticides, more specifically from the class of herbicides. Consequently, effective control and monitoring programs for contamination levels in water must be developed. However, in several countries, including Brazil (one of the largest consumers of pesticides in the world), these programs still need to be implemented more effectively. Few studies of this type have been carried out and legislation on the subject is not updated and unclear, further an increase in the risk of exposure for the agricultural population (Neto and Sarcinelli, 2009).

One of the most common non-selective, systemic pesticides used to keep weeds out of crops is glyphosate, or N-phosphonomethyl glycine, a weak organic acid, which is one of the "active ingredients" of pesticide formulations. To increase its solubility, it is usually formulated as the isopropylamine salt (C6H17N2O5P). Surfactants (e.g., polyoxyethylene amine) and ingredients considered inert are added to some formulations. Along with being utilised in wetlands and aquatic systems, this herbicide is also employed in forestry, home, and urban applications to eradicate illicit crops and control invasive species. Glyphosate has a high degree of mobility in water, which is influenced by both climate and its physicochemical properties. While its low availability in living organisms (log Kow < 0) is determined by its physicochemical properties, it can enter indirectly through the ingestion of glyphosate adsorbed on suspended particulates, and its bioavailability can even be increased by the presence of surfactants or biodegradation (AMPA metabolite) (Villamar-Ayala et al., 2019). Its peculiar chemical properties compared with other pesticides need be considered. Populations can be exposed through different routes and sources, and farmers, in addition to the environmental exposure experienced by the population in general, also have occupational exposure, which can contribute to a significant

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increase in the body burden and, consequently, the risk of toxic effects (Health Canada. CANADÁ, 1987; Amarante Junior et al., 2002; Villamar-Ayala et al., 2019).

Continuous application of glyphosate can cause disturbances in aquatic/terrestrial environments; therefore, it is critical to control its usage and make sure that none of its formulations are dangerous when used over an extended period. It is necessary to consider the actual cell impact of glyphosate-based herbicide residues in food, feed, or the environment, and to discuss their categorisation as carcinogenic, mutagenic, teratogenic, or toxic to human systems (Amarante Junior et al., 2002; Bayona et al., 2022; Novotny, 2022).

One aspect of glyphosate's toxicity to mammals that is often disregarded is its effect on cytochrome P450 enzymes. Also, the herbicide amplifies the deleterious consequences of additional food-borne chemical residues and environmental pollutants. Its detrimental effects are subtle and appear gradually over time as the toxicity can be seen all over the body's cellular systems (Samsel and Seneff, 2015). In 2015, the International Agency for Research on Cancer (IARC) categorised glyphosate as probably carcinogenic to humans (category 2 A), citing the limitations of evidence in humans despite its sufficiency in animals (IARC INTERNATIONAL AGENCY FOR RESEARCH ON CANCER, 2015).

Thus, the present work aimed to analyse glyphosate from aquatic matrices of two cities in the southeast of Brazil – Casimiro de Abreu, Rio de Janeiro state; and Paraguaçu, Minas Gerais state – to discuss the exposure and risk to the rural population of these cities, applying the biomarkers of oxidative stress. To characterize the risk, the hazard quotient was estimated relating the average dairy dose and the reference dose, called maximum acceptable concentration (MAC), in this study.

2. Experimental

2.1. Description of Casimiro de Abreu/RJ and Paraguaçu/MG

In the Southeast Region of Brazil, Rio de Janeiro (RJ) is now among the most urbanized, industrialized, and populous, states and Minas Gerais (MG) stands out in the use of pesticides. In the rural regions of these states, agriculture has great relevance for local economies, which makes it important to assess environmentally and occupationally exposed populations as they are often neglected by public policies. According to data collected in the questionnaire, in rural areas, family farming is predominant.

The high agricultural activity in Casimiro de Abreu/RJ justifies the importance of this research for the region's economic development. The municipality, located in RJ's Baixada Litorânea region, has over 33,500 ha of cultivated land, with family farming dominating the cultivation of banana and cassava (aipim). The municipality is 132 kilometers from the state capital, and according to the Brazilian Institute of Geography and Statistics (IBGE), the city has 46,110 residents, with a declining GDP per capita from 2011 to 2021, the most recent data available. Paraguaçu is located in the southern region of Minas Gerais. With an estimated population of 21,723 in 2021, it has a large rural area and 13 neighbourhoods spread throughout the city. Coffee is the most common crop planted on farmers' properties, along with corn, beans, and soybeans (Ibge Instituto Brasileiro De Geografia E Estatística, 2021).

2.2. Water sampling

Water samples from Casimiro de Abreu/RJ and Paraguaçu/MG, Brazil, were collected from kitchen taps sourced from wells around the houses, which are used for drinking, cooking, and hand and food hygiene. They were chosen based on the risk perceptions of the current study's collaborators, who work in the occupational health and agriculture departments of the two cities. Prior to the pesticide application period, in November 2022, water samples were collected in volunteer homes. A total of 26 samples were collected (n=6, Casimiro de Abreu/RJ and n=20, Paraguaçu/MG). A second sampling was conducted during the application in May 2023, with n=23 from Casimiro de Abreu/RJ and n=20 from Paraguaçu/MG.

The collection procedure adhered to the National Health Surveillance System's (ANVISA) guidelines for collecting, packaging, transporting, receiving, and disposing of samples for laboratory analysis (Anvisa. Agência Nacional de Vigilância Sanitária, 2022); plastic containers were used to prevent the adsorption phenomenon. After collection and transportation, samples were filtered through a 0.45 μM cellulose membrane and stored at -20 ºC for 10 weeks to ensure analyte stability in the matrix, as previously studied.

2.3. Determination of glyphosate from water samples

Sigma-Aldrich® provided standards of glyphosate (N-phosphonomethyl) isopropylamine salt and aminomethylphosphonic acid (AMPA) with purity levels of 97%. All solvents were AR grade. The Milli-Q water system produced ultra-pure water (Millipore®).

Samples were filtered through 0.45 μm cellulose acetate filters and dried in a kiln at 65ºC to start the concentration process. After, the dried was reconstituted with deionized water and derivatized, according to Le Bot et al. (2002). A volume of 3 mL sample was reacted with 0.5 mL of borate buffer (0.05 mol/L) and 500 μL of 9-fluorenyl-methoxycarbonyl chloroformate (FMOC-Cl) solution (1 g/L), under agitation at room temperature for 1 h. The samples were washed with 2 mL diethyl ether and 50 μL of the aqueous phase was injected in the liquid chromatography (Le Bot et al., 2002).

Glyphosate and AMPA were analysed using a high-performance liquid chromatography system (HPLC) from Shimadzu® (Kyoto, Japan), composed of SCL-10A controller, SIL 10-AF automatic injector, LC-10AT pump, CTO-10AS column oven and fluorescence detector (FLD), RF10A. The separation was performed based on Wang et al. (2016), using the C18 column (150 mm×4.6 mm; 5 μm), Chromos®, at 40ºC, and a mobile phase constituted by 5 mmol/L ammonium acetate (pH 9.0) and MeOH, 70:30, v/v. The FLD was set at 265 nm (excitation) and 315 nm (emission) (Wang et al., 2016).

The method was validated based on the guidelines of Ministry of Agriculture, Livestock and Supply (MAPA) ([Mapa] MINISTÉRIO DA AGRICULTURA PECUÁRIA E ABASTECIMENTO, 2015). Linearity, selectivity, limit of quantification (LOQ), accuracy and precision were tested.

Linearity was established through the analytical curve. Blank water samples were fortified (1, 10, 50, 100 e 500 μg/L). All analyses were performed in quintuplicate. Linearity was assessed with the determination and correlation coefficients, calculated from the intra-assay analytical curves. The presence of discrepant observations (outliers) was evaluated in all analytical response values (peak area). Selectivity was verified from the blank sample chromatograms. The LOQ was lowest concentration with precision and accuracy. The intra-assay precision (repeatability) and accuracy were expressed, respectively, as the relative standard deviation (RSD) and the relative error (RE). The inter-assay precision (intermediate precision) and inter-assay accuracy were determined for the same concentrations and on two additional consecutive days. All these tests were performed in quintuplicate.

2.4. Blood sampling

Blood samples were collected via venipuncture using vacuum collection tubes without anticoagulants (for serum) from volunteers over the age of 18, who live and work in rural areas of the two cities and are environmentally and/or occupationally exposed to pesticides. Farmers, nonsmokers, and non-alcoholics were invited. We obtained 20 samples from Paraguaçu/MG and 22 from Casimiro de Abreu/RJ. Furthermore, samples were collected from an environmentally exposed pesticide group residing in an urban area (n=20 and n=22, respectively, from Paraguaçu/MG and Casimiro de Abreu/RJ) to ensure the study's statistical significance. Volunteers with comorbidities such as cancer,

which could interfere and bias the results, were excluded from the groups.

The study was approved by Ethics Committees of the Federal University of Alfenas-MG (CAAE 34644620.2.0000.5141) and of National Institute of Cancer (INCA) (CAAE: 64799217.3.0000.5274). Each volunteer's privacy was strictly respected, and all laboratory analyses performed as part of this study were completely free of charge.

2.5. Determination of oxidative stress biomarkers in blood

Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) in serum, which indirectly reflects malondialdehyde (MDA) production through fluorimetry (Sinnhuber and Yu, 1977). Analysis were conducted using a spectrofluorimeter (Cary Eclipse Fluorimeter, Varian®) with excitation and emission wavelengths set at 532 nm and 553 nm, respectively.

Superoxide dismutase (SOD) activity was measured in serum samples using the colorimetric assay developed by (Oyanagui, 1984). The spectrophotometer was set to 550 nm (UV-Vis Spectrophotometer with Peltier Temperature Control, Thermo Scientific®). The enzyme activity was calculated based on the principle that 1 unit of the enzyme produces a 50% inhibition in the reaction. The results were expressed relative to the protein concentration of the sample.

Catalase activity (CAT) was spectrophotometrically in serum samples by monitoring the consumption of H_2O_2 per minute at 240 nm following the method described by (Aebi, 1984). The results were expressed in units (U) per milligram (mg) of protein, where one U corresponds to the activity of the enzyme that hydrolyzes 1 μmol of H_2O_2 per minute.

The determination of total proteins was performed using the Bradford method, as described by (Zaia et al., 1998). This method utilizes Coomassie brilliant blue dye to measure total serum proteins. A standard solution of bovine serum albumin protein (BSA) was used to generate an analytical curve for quantification. The absorbance was measured at 595 nm using a spectrophotometer (the same as SOD).

2.6. Risk characterization

The average daily potential dose (ADD) was estimated for the Brazilian populations studied, as the product of the contaminant concentration (Cmedium, μg/L), intake rate (IR, intake rate of the contaminated water, L-day), exposure frequency (EF), and exposure duration (ED), divided by the product of the body weight (BW) and averaging time (AT, amount of time over which exposure is averaged, for assessing non-cancer risks) (Atsdr AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, 2023), from consuming water contaminated with glyphosate and AMPA (Eq. 1):

$$\mathrm{ADD}(\mu g \Big/ Kg - day) = \frac{\text{Cmedium x IngR x EF x ED}}{\text{BW x AT}} \quad (1)$$

Hazard Quotient (HQ) was estimated by the ADD divided by MAC (Maximum Acceptable Concentration, a reference value), which was calculated according the proposed by Bayona et al., (2022) (Eq. 2):

$$\mathrm{MAC}(mg \Big/ L - day) = \frac{\text{Tolerable daily intake (TDI) x BWx 20\%}}{\text{Water consumption (L} - \text{day)}} \quad (2)$$

The HQ allows for the assessment of the risk of pesticide exposure. When the HQ is less than one, it is considered a low health risk (Health Canada. CANADÁ, 1987; Bayona et al., 2022).

2.7. Statistical analysis

Data were analyzed using the BioStat® program, version 5.0 (AnalystSoft Inc.). To assess data normality, the Shapiro-Wilk test was used. For the oxidative stress, to performed comparisons of means between two groups, the Mann-Whitney test was applied. The significance level chosen for the study was 5%.

3. Results and discussion

3.1. Glyphosate from water samples

Exposure assessments of the pesticides are one of the essential parameters in evaluating and regulating the suitability of water intended for human consumption. The surveillance and monitoring of these compounds are of significant relevance to Brazilian society, considering not only the toxicity of these substances, but also their high demand in the agricultural sector. Even when water is considered suitable for human consumption, there are a significant number of chemical residues. One can become exposed to contaminants in water through direct ingestion (drinking water) or indirect ingestion (eating foods and beverages prepared with water). It is also possible for accidental ingestion (such as when a swimmer swallows water while swimming), dermal contact (such as when bathing or showering, while swimming, or when wading in surface water), or inhalation to happen (such as when a showerer inhales vapours) (Usepa U.S. Environmental Protection Agency, 2023).

In general, to determine glyphosate by liquid chromatography coupled to a fluorescence detector, even from different matrices, the derivatisation step is highly relevant. FMOC-Cl is a reagent commonly used for this goal. The methods are generally similar at this step, using a basic medium (pH around 9) and excess derivatisation to ensure a reaction with all of the glyphosate and AMPA in the sample, as both analytes lack fluorescent groups susceptible to direct detection (Amarante Junior et al., 2002; Le Bot et al., 2002; Wang et al., 2016).

The range in this study was chosen by current legislation in Brazil which establishes maximum permitted values of pesticides and metabolites in water for human consumption and regulates a value of 500 ppb (μg/L) for the sum of glyphosate and AMPA (MS MINISTÉRIO DA SAÚDE, 2021), which was the upper limit of the linear range. Under these circumstances, the points 1, 10, 50, 100, and 500 ppb were established on the analytical curve to demonstrate linearity, selectivity, precision, and accuracy. The limit of quantification (1.0 ppb) was reliable to quantify the analytes in the collected samples (Table 1) and satisfactory to discuss risks from exposure.

Glyphosate is water soluble (12 g/L at 25°C) and stable to hydrolysis in water, but it undergoes rapid microbial degradation in natural surface waters. Photolysis can also occur, and strong adsorption to particulate matter may be a factor in glyphosate's removal from water (Health Canada. CANADÁ, 1987; Amarante Junior et al., 2002). As can be observed in Table 2, comparisons of water sample medians collected in the residential taps of homes in the rural area of Casimiro de Abreu/RJ and Paraguaçu/MG before and during herbicide application showed a significant difference, $p < 0.05$, for glyphosate plus AMPA. These findings suggest that such samples are environmentally contaminated, since

Table 1
Realiability of the method developed to determinate of glyphosate and AMPA in drinking water in the range studied.

Reliability parameter	Glyphosate	AMPA
Range (ppb)	1–500	
a	2868510	
b	11457831	
r	0.9984	0.9951
r^2	0.9931	0.9903
LOQ (ppb)	1.0	1.0
Precision (% relative standard deviation)	9.4–11.5	14.2–19.7
Accuracy (% relative standard error)	4.7–10.7	9.4–14.2

Note: a= angular coeficient; b= linear coeficient; r= correlation coeficient; r^2= determination coeficient; LOQ (limit of quantification).

Table 2
Range, median and standard deviation (SD) of the glyphosate plus AMPA concentrations in water samples from residential taps of Casimiro de Abreu/ RJ and Paraguaçu/ MG.

Before the glyphosate application		During the glyphosate application		
Casimiro de Abreu/RJ				
Number of samples	**Range; median (± SD) (ppb)**	**Number of samples**	**Range; median (± SD) (ppb)**	***p* value**
6 analysed 1 quantified	< LOQ[1] to 17.07; < LOQ[1] (± 6.87)	23 analysed 20 quantified	< LOQ[1] to 29.74; 3.26 (± 6.49)	0.034
Paraguaçu/ MG				
Number of samples	**Range; median (± SD) (ppb)**	**Number of samples**	**Range; median (± SD) (ppb)**	***p* value**
20 analysed 3 quantified	< LOQ[1] to 65.11; < LOQ[1] (± 15.70)	20 analysed 14 quantified	< LOQ[1] to 12.58; 1.78 (± 2.92)	<0.001

Notes: 1. < LOQ: samples with the analyte detected but not quantifiable by the method; 2. Mann Whitney test.

the analyte, due to its physical-chemical characteristics, is an important contaminant of surface water supplying homes, and an environmental degradation byproduct of glyphosate in its metabolite, which can be also accumulated in this environmental context (Villamar-Ayala et al., 2019).

Glyphosate causes aquatic contamination because it can enter surface waters either after direct use near aquatic environments or by runoff or leaching from terrestrial applications. The transportation of glyphosate and AMPA from agricultural areas in Mississippi (USA) to seven streams located in low-lying hydrocarbon areas was assessed by Coupé et al. (2012). Nearly all samples (87%) presented the analytes, with their concentrations ranging from 0.08 to 73 ppb. The authors noted that the watersheds most at risk for the offsite transport of glyphosate are those with high application rates (Coupé et al., 2012).

Table 2 shows that when glyphosate formulations are used in Casimiro de Abreu/RJ, 87.0% of the samples are contaminated with the herbicide and its metabolite, and the median is statistically different from the value obtained prior to application. In the Paraguaçu/MG scenario, the data are similar during the application, with 70.0% of the drinking water samples containing glyphosate plus AMPA at levels higher than the LOQ of the method used.

3.2. Oxidative stress markers

An analysis of MDA, SOD, and CAT was carried out by Abarikwu et al. (2015) in the livers of Wistar rats that were exposed to a commercial formulation containing glyphosate. The analysis demonstrated that MDA and CAT increased, while SOD activity remained unchanged (Abarikwu et al., 2015). Novotny (2022) discussed that there is a global emergency in the review of regulations for pesticides. At the moment, regulators may only assess the 'active ingredient,' glyphosate, and ignore the toxicity of the formulants (Novotny, 2022).

According to Beuret et al., 2005, lipid peroxidation and other metabolic alterations brought on by glyphosate may be sensitive to changes during development and pregnancy. The current study examined the effects of oral exposure to 1% glyphosate on antioxidant enzyme systems and lipoperoxidation in the liver and maternal serum of pregnant rats, as well as in the term fetuses at 21 days of gestation. The findings imply that glyphosate consumption causes excessive lipid peroxidation, which overwhelms the antioxidant defence systems of the mother and fetus (Beuret et al., 2005).

Kwiatkowska et al. (2014) evaluated the toxic potential of glyphosate, its metabolites (aminomethylphosphonic acid [AMPA] and methylphosphonic acid) and its impurities (N-(phosphonomethyl) iminodiacetic acid [PMIDA], N-methylglyphosate, hydroxymethylphosphonic acid, and bis-(phosphonomethyl)amine), studying the effect of those compounds on the formation of reactive oxygen species (ROS). The authors reported that most of the investigated compounds induced ROS formation from 0.25 mmol/L, while N-methylglyphosate caused the effect from 0.5 mmol/L (Kwiatkowska et al., 2014).

The results obtained by Cattani et al. (2017), showed that the chronic exposure of pregnant rats to drinking water, containing glyphosate, produced oxidative stress and glutamate excitotoxicity in the hippocampus when rat offspring were exposed to glyphosate for 60 days. The mechanism of action discussed was a decrease in glutathione levels. Induction of oxidative damage to immature rat hippocampal cells was also associated with a combined decrease in the activity of important antioxidant enzymes such as SOD, glutathione S-transferase, and glucose-6-phosphate-dehydrogenase (Cattani et al., 2017).

The data presented in this study are innovative since results from human exposed to glyphosate are still sparce. We evaluated several parameters related to monitoring and health surveillance in the two Brazilian populations. The populations studied were like those observed in previous Brazilian studies with farmers (Silvaério et al., 2017; Machado et al., 2021; Costa et al., 2023; Marciano et al., 2024). The range of ages was 45–60 years, with an educational profile up to elementary school. There were more than 10 years of exposure to pesticides, and formulations containing glyphosate are the most used. The farmers are considered settled in their place of residence and do not receive periodic technical visits from professionals involved in training and promoting technical education.

Table 3 and Fig. 1 show the results of the mean, standard deviation, and median of the oxidative stress biomarkers evaluated in this study, comparing the levels in farmers exposed to glyphosate (directly exposed) with an environmentally exposed group. Oxidative stress is a biological process in which an imbalance occurs between the production of reactive oxygen species (ROS) and the body's ability to neutralise them through antioxidants. To evaluate the level of oxidative stress in the studied groups, serum lipid peroxidation was measured indirectly by

Table 3
Mean, standard deviation (SD) and median of the oxidative stress biomarkers from workers directly exposed to pesticides and environmental exposed group of Casimiro de Abreu/ RJ and Paraguaçu/MG.

Stress oxidative biomarker	Directly exposed group (mean ± SD; median)	Environmental exposed group (mean ± SD; median)	*p* (Mann Whitney test)
Casimiro de Abreu/RJ	n=22	n=22	
SOD (U/mg protein)[1]	0.060 ± 0.056; 0.037	0.234 ± 0.051; 0.222	<0.001
CAT (U/mg protein)[2]	0.441 ± 0.238; 0.395	14.736 ± 4.155; 14.840	<0.001
MDA (U/mg protein)[3]	3.557 ± 0.960; 3.375	0.102 ± 0.047; 0.100	<0.001
Paraguaçu/MG	n= 20	n= 20	
SOD (U/mg protein)[1]	0.151 ± 0.033; 0.157	0.233 ± 0.045; 0.222	<0.001
CAT (U/mg protein)[2]	10.100 ± 3.410; 9.600	14.500 ± 3.240; 14.700	<0.001
MDA (U/mg protein)[3]	0.137 ± 0.053; 0.135	0.093 ± 0.0496; 0.080	<0.001

Notes: 1. SOD= Superoxide dismutase; 2. CAT= Catalase activity; 3. MDA= malondialdehyde.

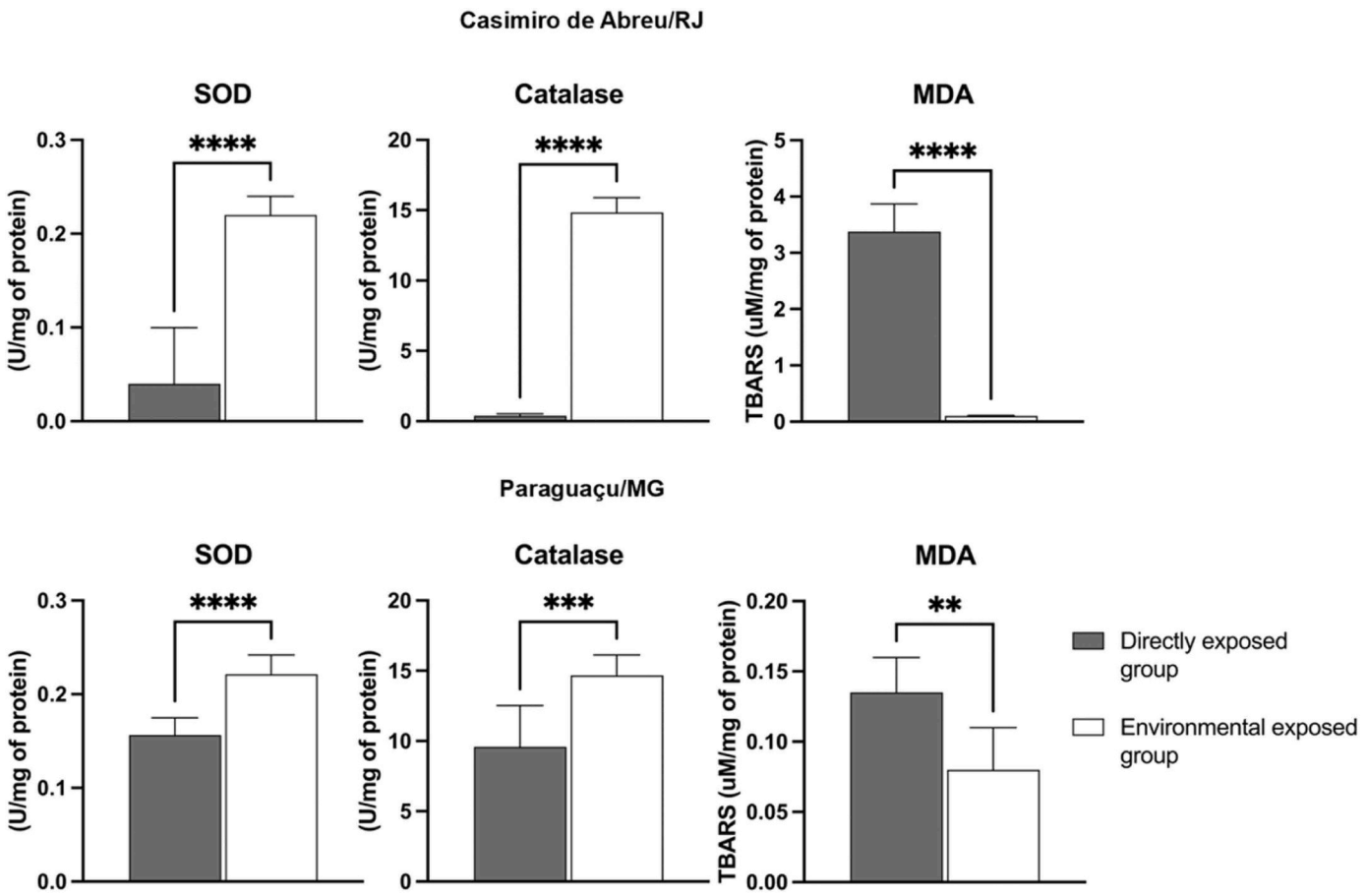

Fig. 1. - Representation of the differences of the oxidative stress biomarkers from workers directly exposed to pesticides and environmental exposed group from Casimiro de Abreu/ RJ and Paraguaçu/MG, Brazil.

determining MDA production. The SOD and CAT enzymes are part of the antioxidant defence mechanism, working to neutralise excess production of oxidising species.

The results demonstrated that the directly exposed workers have lower levels of SOD and CAT enzyme activity compared to the environmentally exposed group (urban residents). However, lipid peroxidation assessed from MDA is increased in the occupationally and environmentally exposed group. Thus, it is possible to indicate that there isn't homeostasis in the organic oxidative processes. This can be caused by exposure to pesticides, indicating reduced levels of defence to the chemicals' cellular action.

Peixoto (2005) demonstrated the ability of Roundup® to impair mitochondrial bioenergetic reactions through changes induced in mitochondrial respiration. He demonstrated that Roundup® affects the electron transfer redox chain at the level of complexes II and III. Electron leakage in the respiratory chain leads to the unregulated formation of reactive oxygen species responsible for cellular damage (Peixoto, 2005). Moreover, Jasper et al. (2012) correlated the production of oxidising species with haematological changes resulting from lipid peroxidation. The exposure of rats to glyphosate, even at low doses and for a short period of time, induced serious hepatic and haematological damage caused by increased oxidative stress. The increase in the production of oxygen species with the reduction in the activities of antioxidant enzymes contributed to the advancement of cellular injuries (Jasper et al., 2012).

Only a few studies have explored redox imbalances and oxidative stress biomarkers in Brazilians exposed to pesticides. Jacobsen-Pereira et al. (2018) discovered increased lipid peroxidation and related genotoxicity effects in rural workers exposed to pesticides yet saw no significant change in CAT activities between exposed and non-exposed groups (Jacobsen-Pereira et al., 2018). In the same way, this study shows that the reduction in the activity of antioxidant enzymes in directly exposed workers leads to a weaker defence against reactive oxygen species. In addition to these findings, there is concern about this population of self-employed workers, who only demand for care/treatment when they are already sick (Silvério et al., 2020). Santos et al. (2021) observed that living in rural areas compared to urban ones was linked to disruptions in glutathione peroxidase activity and a negative correlation with glutathione-S-transferase activity in response to insecticide use. However, they found no statistical difference in CAT and SOD activities between the two groups, diverging from our findings (Santos et al., 2021).

In contrast, both Sasso et al. (2021) and Marciano et al. (2024) identified a redox imbalance characterised by elevated lipid peroxidation in similar populations. Specifically, Sasso et al. reported significantly decreased activities in CAT, SOD, glutathione, glutathione reductase, glutathione peroxidase, and glutathione S-transferase among rural workers. Marciano et al. found reduced CAT and SOD activities in workers exposed to triazole fungicides in Minas Gerais' southern region compared to urban volunteers not occupationally exposed to pesticides (Sasso et al., 2021; Marciano et al., 2024).

Glyphosate induced inflammatory pathway activation is associated with increased ROS production and impaired antioxidant defences. Evidence shows that the increase in intracellular Ca^{2+} levels and the oxidative stress induced by glyphosate trigger the activation of pro-inflammatory and pro-apoptotic pathways (Batista et al., 2023). Sasso et al. (2021) identified a significant increase in IL-8 expression, which is a pro-inflammatory interleukin, in groups exposed to pesticides, in

addition to demonstrating an imbalance in antioxidant enzymes (Sasso et al., 2021). In addition, an assessment of liver toxicity in mice exposed to glyphosate demonstrated that excessive ROS activate the complement and coagulation cascade pathways, increasing the release of inflammatory mediators and the aggregation of inflammatory cells (Qi et al., 2023).

All these findings demonstrate that oxidative stress may trigger the release of inflammatory mediators, contributing to tissue injury and dysfunction in the target organs of oxidative stress.

3.3. Risk characterisation

A rural worker can be exposed to pesticides both during application and by consuming contaminated food or water. Similarly, populations living near pesticide-treated areas may consume contaminated water or food, as well as inhale substances that will eventually be useful in the air. Furthermore, the same individual may be exposed to more than one type of pesticide, even if only through one route, creating a high-risk situation (Neto and Sarcinelli, 2009). In this context, the studied populations were interviewed, and questionnaire data (body weight, water consumption, exposure duration, and averaging time) were used to calculate the risk.

A TDI for glyphosate was established as 0.03 mg/kg bw per day, where 3.0 mg/kg bw/ day was the No Observed Adverse Effect Level (NOAEL) for reduced body weight gain in a two-year rat feeding/ oncogenicity study and 100 is the uncertainty factor (10 for interspecies variation and 10 for intraspecies variation in an adequate long-term study) (Bayona, 2022). In the present study, the glyphosate MAC was 0.21 mg/L-day (210 μg/L-day), assuming 70 Kg as the average the body weight of the populations studied, 20% as the contribution of glyphosate intake by drinking water (Bayona et al., 2022), related with the value of 2 L-day, as consumption of drinking water by a Brazilian adult.

Table 4 shows that the HQ was calculated using the sum of glyphosate and AMPA in the worst-case scenario, as determined during herbicide application in the two cities. The HQ calculated for Paraguaçu/ MG indicated a low risk of developing non-carcinogenic effects (HQ $<$ 1). Casimiro de Abreu/RJ non-carcinogenic quotient exceeded the acceptable level, and adverse health effects were probable, as the calculated results showed HQ $>$ 1 (Bayona et al., 2022). However, this study reveals potential effects resulting from pesticide exposure, as evidenced by biomarkers of oxidative stress. Furthermore, it is worth highlighting the one sample from Paraguaçu/MG with the maximum concentration determined in the present study (65.11 ppb) before herbicide application. This suggests the significance of considering potential variations in substance concentrations, which can be influenced by many factors. Bayona et al. (2022) determined values as low to moderate risk by the HQ, as the concentrations found in the drinking water supplied to the metropolitan area of Cúcuta had values of 216 and 204.5 ppb in the samples analysed (Bayona et al., 2022).

The conventional systems currently used for water purification are insufficient to remove these pesticides. It is important to note that herbicide concetrations increase to the extent that certain worker practices, such as washing tools and containers used in the preparation and dilution of agrochemicals, or even acid spill cleanup, are frequently carried out. Nevertheless, these farmers dont receive suficient training and underestimate the risks of the long-term toxic effects. Attention must be given to both knowledge of causal factors, and trends in detecting concentrations of hazardous chemicals that occur in drinking-water are of concern because effects arising from sequences of exposure over a short period may indicate that significant toxic effect may arise in the future.

Numerous aquatic organisms may be affected both acutely (primary producers and primary consumers) and chronically (secondary consumers), according to ecotoxicological research. A greater toxicity in marine organisms compared to freshwater organisms is the subject of multiple conclusions in this regard. Furthermore, throughout the food chain, its involvement can happen at different levels. Ultimately, the formulations used in commercial products have been found to have the greatest impact on aquatic environments (Villamar-Ayala et al., 2019).

Analytical data referring to the levels of glyphosate and AMPA in water intended for human consumption, obtained by the Ministry of Health through Water Quality Surveillance (SISAGUA) over a period of nine years, didnt demonstrate the presence of concentrations higher than the established maximum allowed values (MS MINISTÉRIO DA SAÚDE, 2023). However, it is observed that water is a source of exposure to glyphosate, significantly contributing to the increased risk of toxic effects (Novotny, 2022). This is corroborated by the data presented in Fig. 2 based on the questionnaire given to the volunteers, in which it was possible to observe that most of the rural population under observation consumes water from wells and springs.

Studies showed that glyphosate-based herbicide formulations are harmful to the environment and human health; therefore, it is very important to have the attention of the regulatory authorities that implement programs to assure the health of both ecosystems and populations. To a reliable risk assessment, the time and frequency of exposure must be considered, corroborating the importance of exposure monitoring programs. The exposure tends to be chronic, as the glyphosate molecule has polar characteristics making it soluble in water. However, once it reaches aqueous media, its half-life ($t_{1/2}$) can vary from 63 days (shallow waters) up to 70 days (pond water) at concentrations lower than 2 g/mL and at neutral pH (7.2) (Amarante Junior et al., 2002; Villamar-Ayala et al., 2019).

Furthermore, if the results of the present study (Table 2) were compared with the more restrictive European Union legislation, which recommends 0.1 ppb as the maximum allowable concentration in drinking water (5000 times lower than the limit proposed in Brazil),

Table 4
Hazard quotient of the high level determined of glyphosate plus AMPA, in drinking water samples, from Casimiro de Abreu-RJ and Paraguaçu/MG, Brazil, during the application of the formulations containing the herbicide.

	Casimiro de Abreu/ RJ	Paraguaçu/ MG
Contaminant concentration (Cmedium, μg/L)	29.74	12.58
Ingestion rate (IngR, L)[1]	2	
Exposure frequency (EF, day)[2]	365	
Exposure duration (ED, years)[1]	70	
Body weight (BW, Kg)[1]	70	
Averaging time (AT, years)[1]	70	
Average daily dose (ADD, μg/Kg-day)[2]	310.1	131.1
Maximum Acceptable Concentration (MAC, μg/L-day)	210	
Hazard Quotient (HQ)	1.5	0.6

Notes: 1. In the context of this study, for Brazilian rural populations; 2. Atsdr AGENCY FOR TOXIC SUBSTANCES AND DISEASE REGISTRY, (2023).

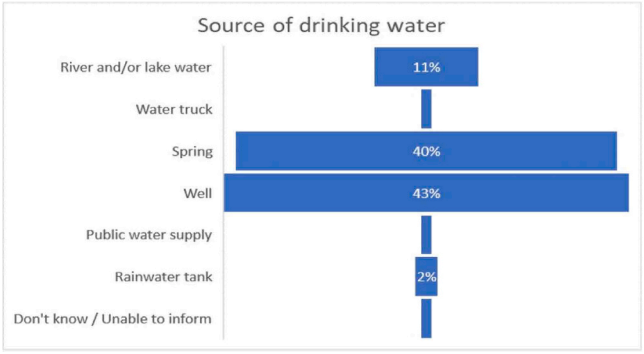

Fig. 2. - Percentage of drinking water sources consumed by the rural population from Casimiro de Abreu/ RJ and Paraguaçu/MG, Brazil.

several samples would be considered unsuitable for consumption (Eu EUROPEAN UNION, 2020).

Attention must be given to both knowledge of causal factors and trends in the detected concentrations of hazardous chemicals that occur in drinking-water. These are of concern because effects arising from sequences of exposure over a short period may indicate that significant toxic effects may arise in the future, since the conventional systems currently used for water purification are insufficient to remove the pesticides. Even though most countries require toxicity tests with aquatic organisms to register new pesticides, studies must be conducted with tropical countries' local species to assess bioaccumulation and impact analysis on the aquatic environment and on the chain food.

Alvarez et al. (2023) discussed that there are some associated data gaps that are relevant to calculate limit values and to assess the risks of glyphosate exposure, as impurities demonstrated the potential for clastogenicity in an *in vitro* chromosome aberration test that was not properly followed up *in vivo*, so an assessment of the reference specification cannot be finalised. Its clastogenic potential must be clarified before any conclusions about the maximum level of this impurity in any reference specification can be made; due to incomplete data on the amount of residues in rotational crops, a consumer dietary risk assessment could not be completed (Alvarez et al., 2023).

In this study, the non-carcinogenic risks for rural exposure to glyphosate and AMPA in Casimiro de Abreu/RJ and Paraguaçu/MG could be underestimated because only the drinking water exposure route was analysed. Thus, more exposure monitoring programs, such as urinary biomonitoring and monitoring of foods consumed in these regions, are needed in future studies for accurate and precise estimations of the noncarcinogenic risks of exposure to pesticide formulations and mixtures among rural farmers and inhabitants.

Considering that the organisation of the populations studied is mainly family farming characterised by the proximity of housing to fields, they can become exposed to contaminants by multiple sources and by all main routes of exposure. This is a critical public health problem and governmental negligence, in terms of lack of public policies for promoting health, increases the exposure and the risk of susceptible groups, such as children and pregnant, elderly, and sick people.

4. Conclusions

A reliable analytical method was used to detect and quantify glyphosate and AMPA in drinking water, and despite the low risk, the results revealed that the population is exposed to the herbicide. The levels of glyphosate and AMPA found in the samples were less than Brazil's recommended limit, which is 5000 times less restrictive than the European Union's recommendation. When the results are compared to the latter, the water is considered unsafe for consumption because it is an important source of chronic environmental exposure for the population of Casimiro de Abreu/RJ and Paraguaçu/MG, emphasizing the need to discuss Brazilian regulations and allowable levels.

The current study's findings suggest that the rural population may be more susceptible to current toxic effects due to factors such as multiple sources of exposure and longer periods of pesticide exposure. Agricultural workers, both occupational and environmentally exposed, had an increase in lipid peroxidation and a decrease in defense enzymes. Our human data contribute to the discussion of revising pesticide formulation regulation practices while also demonstrating the importance of implementing monitoring and biomonitoring programs to reduce exposure.

Finally, our critical results provide information that corroborates and supports discussions on the topic of sustainable food production, using non-polluting, economically efficient, and safe processes and systems for rural workers, communities, and consumers, to prevent pesticide acute intoxication and adverse health effects in situations of chronic exposure.

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CRediT authorship contribution statement

Isarita Martins: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Geovana Sousa Oliveira:** Methodology, Investigation. **Naiane Silva Cardoso:** Writing – review & editing, Methodology, Investigation. **Fernanda Borges de Araújo Paula:** Writing – review & editing, Resources, Funding acquisition. **Marcia Sarpa:** Writing – review & editing, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Rafaella Ferreira Nascimento Nunes:** Writing – original draft, Methodology, Investigation, Formal analysis. **Luiz Paulo de Aguiar Marciano:** Writing – original draft, Methodology, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Research paper

Assessment of cellular damage with cytome assay among environmental/occupational triazole

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ABSTRACT

The use of triazole fungicides is common in Minas Gerais, Brazil. However, the risk arising from excessive and often unprotected exposure can be harmful to farmers. Therefore, we evaluated volunteers, exposed to triazole fungicides for cellular damage caused by this pesticide. In the buccal micronucleus cytome assay (BMCyt), cells were analyzed. Urinary triazoles were analyzed by the Liquid-Liquid Extraction coupled with Gas-chromatography/mass-spectrometry (LLE-GC/MS). Statistical differences were found for all cell types evaluated in residents of rural areas (n = 145). Analysis of variance showed statistical difference in kariolytic and pyknotic cells, between the groups of men and women living in rural areas, with higher incidence in the male group. Likewise, higher concentrations triazoles in urine samples in the male group were observed. Greater cellular damage suggests increases in DNA damage, chromossomal instability and cell deaths. The results showed the urgency of the public management with the implementation of measures to minimize the pesticides exposure.

1. Introduction

In recent years, Brazil has become one of the main consumers of pesticides in the world, particularly in the agricultural sector [1]. In the southern region of Minas Gerais state, coffee cultivation is a major pillar of the local economy. To maintain crop productivity, male farmers commonly apply pesticides, potentially exposing themselves occupationally through spraying and environmentally due to their proximity to the crops [2,3]. Meanwhile, women, even though they don't participate in spraying, may still be exposed to pesticides environmentally or indirectly when handling their husbands' clothes. There are several classes of pesticides existing and used by farmers. Triazole fungicides are commonly used to prevent or control pests in crops, especially in coffee plantations, the predominant crop in this region of southern Minas Gerais, Brazil [4].

As the main, if not the only source of income for farmer's cultivation and marketing of coffee, these farmers commonly use pesticides as a tool to control or prevent pests that can harm fruit productivity and thus compromise sales [5]. However, the inappropriate use, the lack of adequate training on the safety handling, associated with the less knowledge of the existing risks by the farmers in the handling of the substances are gaps that can lead to potential exposure [6]. Despite being relatively efficient against fungi, triazole fungicides have low specificity, being able to interact with different organisms to which they come in contact including humans [4].

Research has shown that exposure to triazole fungicides is correlated with hormonal changes and the ability to cause cellular genotoxicity. As a result, this exposure can lead to the development of severe disorders, including hormonal effects and the emergence of various diseases [7]. There aren't data about carcinogenicity of these xenobiotics. To assess possible exposure, exposure and effect indicators are commonly used, involving the detection of pesticides in biological materials [8]. In addition, evaluating specific biological markers is crucial in biomonitoring to detect potential changes resulting from exposure [9]. Therefore, a proper evaluation of the farmer's health can be conducted, allowing for the implementation of necessary corrective or preventive measures based on the findings [9].

Buccal micronucleus cytome (BMCyt) assay has been used in recent

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studies as an important tool in the assessment of exposure to chemical agents [9,10]. Previous studies used only the presence of MN as an indicator of the effect [11,12], however, as it is currently known that other cellular changes such as binucleated, karyolitic, karyorrhecyic and pycnotic cells may be related to exposure to chemical agents, especially pesticides [9]. In the systematic review study carried out by Ref. [13]; it was found that evaluating the presence of MN in buccal cells, in lymphocyte and the comet assay were used by researchers who evaluated the exposure to pesticides of volunteers in Brazil between the years from 1980 to 2021. [14] utilized the cytome assay to evaluate tea garden workers exposed to pesticides, revealing greater cellular damage in the pesticide-exposed group compared to the control group.

[9] evaluated 94 individuals exposed to organophosphates using the cytome assay. A higher incidence of cell damage was found in all types evaluated (binucleated, karyolytic, karyorrhectic, condensed chromatin, pyknotic and MN), with statistical difference for cells with condensed chromatin and karyolytic cells in the exposed group. In another study in India, the cytome assay was employed as a bioindicator of effect for 75 individuals using sadagura, a unique smokeless tobacco prepared in southern Assam province of North-East India, was evidenced. Statistical differences were observed in the exposed group, indicating a higher incidence of MN and binucleated cells compared to the control group. Although no statistical difference was found, higher incidences of karyolytic, karyorrhectic and pyknotic cells were found in the exposed group when compared to the control [15].

Understanding the different types of cellular alterations and their possible causes and risks is crucial for adequately monitoring individuals, offering them greater attention and care for their health [10].

Therefore, we evaluate individuals living in the rural zone of the south Minas Gerais, Brazil, exposed to triazoles by occupational/environmental sources. To investigate potential correlations between exposure and cellular damage, we applied the BMCyt assay as an effect indicator. This analysis sought to identify an increased risk of disease development related to the genotoxicity of triazole pesticides for this population. Urinary triazole were used results as an internal dose bioindicator.

2. Materials and methods

2.1. Study design and sampling

The study was carried out in the southern region of Minas Gerais, Brazil, with n = 145 volunteers of both genders residing in the rural area. As inclusion criteria, male volunteers who work in coffee plantations, being responsible for the application of triazole fungicides, as well as women who live in the rural area and have no direct contact with triazole fungicides through application, were invited. As a control group (n = 50), healthy men and women residing in the urban area, who perform functions not related to the field and who self-reported not having contact with triazole fungicides, were required. Only individuals over 18 years of age were invited to participate in the research, as an exclusion criteria individuals with a recent or past history of cancer, were excluded from the study. Informed consent was obtained from each volunteer by signing an informed consent form and this study was approved by the Ethics Committee of the Federal University of Alfenas-MG (CAEE 34644620.2.0000.5141).

On December 11th and 12th, 2021, during a period of intense application of triazole fungicides in the region of this study, urine samples and swabs from the oral mucosa of the volunteers were collected. A questionnaire (supplementary material) was also applied to obtain relevant information, such as smoking, alcoholism, time of exposure to triazole fungicides in years, recent exposure to X-rays, among others. All samples were transported at 8 °C. Urine samples was stored at −26 °C until analysis. Buccal cells were prepared on the same day of collection and stored in cases at room temperature.

2.2. Determination of fungicides triazoles in urine by GC-MS

The determination of urinary triazoles was performed using the method adapted from Ref. [4] employing gas chromatography coupled with mass spectrometry (QP 2010 Plus, Shimadzu®), being able to detect the fungicides: cyproconazole, epoxiconazole, metconazole, propiconazole and triadmenole. The extraction process being optimized and validated according to national and international validation guide [16–18]). For the urine extraction process, in a falcon tube with a capacity of 15 mL, 100 μL of the enzyme β-glucuronidase from *Helix Pomatia* (Sigma-Aldrich®) were added to 1 mL of urine and incubated at 38 °C for 12 h 20 μL of internal standard (Tebuconazole-*tert*-butyl-d9, Sigma-Aldrich®), followed by 2 mL of dibasic sodium phosphate buffer (Vetec®) pH 7, 1 mL of acetonitrile (Dinâmica®) and 200 μL of Toluene (Química®), which was the extracting solvent were added. Then, the sample was mixed for 1 min and centrifuged at 1650g for 5 min 200 μL of the supernatant was transferred to an ependorff, and taken to a dryer (LABCONCO Centrivap®) in 20 min at room temperature. The dry extract was resuspended in 100 μL of Toluene, and 2 μL were injected into the chromatographic system under the same conditions described by Ref. [4].

2.3. BMCyt assay

The BMCyt assay was performed as described by Refs. [9,19]. Buccal swabs using cytobrush on both sides of the volunteer's cheeks were gently rubbed. After collection, the material was immersed in a falcon tube containing 5 ml of saline solution of sodium chloride (0,9% w/v) and transported to the laboratory. After mixing, the cytobrush was discarded and the solution was centrifuged for 5 min at 750g. In addition, 4 mL was discarded, and 5 mL was made up again with saline solution and centrifuged under the same conditions. After performing the centrifugation process three times, a fourth centrifugation was performed using 4 mL of Carnoy's fixative solution, which is a solution of acetic acid with methanol in the proportion of 1:3. After centrifugation, the supernatant was discarded, leaving only 1 mL of the solution in the tube. The cell pallet was gently suspended, placed on the three slides with the aid of a pipette and dried at room temperature.

Acridine orange, at a concentration of 0.001% in water, was used to stain the slides for 5 min. The stained slides were then rinsed in distilled water for 2 min and covered with a coverslip. The slides were analyzed under a fluorescence microscope (Leica® microsystems) with a blue filter and magnification of 400.

Cellular damages such as binucleated cells (cell containing two nuclei in the cytoplasm), karyolitic cells (cell without nucleus in the cytoplasm), karyorrhectic cells (cell with disintegrating nucleus), cells with MN (cells with one or more fragments of nucleus in the cytoplasm) and pyknotic cells (cells with reduced nucleus) were analyzed and scored as per [20]. Two slides per volunteer were analyzed, where a total of 1000 damaged cells were scored per slide and the arithmetic mean was calculated to represent the frequency of each alteration, for the application of statistical tests between groups.

2.4. Statistical analysis

Data were analyzed using the GraphPad Prism program, version 9.3.0 (Dotmatics). Data are demonstrated as mean ± standard deviation (SD) and median with 95% confidence interval. Normality was assessed using the Shapiro-Wilk test. Non-parametric data were evaluated using Kruskal-Wallis tests, with post hoc being Dunn's test for multiple comparisons. For comparison only between two groups, the Mann-Whitney test was applied. The significance level to be adopted is 5%.

3. Results

In this study, volunteers living in rural areas were evaluated,

Table 1
General characteristics of the study volunteers.

Variables	Rural residents (n = 145)	Urban residents (n = 50)
Gender		
Male (n)	90	25
Women (n)	55	25
General characteristics		
Age of volunteers (mean)	46.90 ± 12.25	25.10 ± 6.70
Education in years (mean)	6.91	17.68
Smoking habit (n)	21	08
Alcoholism (n)	20	31
Recent X-ray exposure* (n)	14[a]	16[a]
Years of exposure to triazoles fungicides (mean)	19.40	none
Mode of application to triazole fungicides		
Costal pump (n)	40	none
Tractor with cabin (n)	39	none
Tractor without cabin (n)	11	none
System Featuring Changes (%)		
Cardiovascular	29.65	6.00
Central Nervous	48.96	48.00
Digestive	40.00	32.00
Respiratory	28.96	28.00
Hearing	21.37	8.00
Skin and mucous membranes	18.62	0.00
Urinary triazoles μg L^{-1} (mean)	12.41	none

Note: none = not applicable.
*Recent exposure to X-rays = less than 60 days.
[a] Values with equal letters did not show significant difference in the BMCyt assay (Mann-Whitney, 5% significance).

including 145 individuals directly (applying triazole to their coffee plantations) and indirectly exposed (living in rural area but not occupationally exposed to fungicides). As a control group (n = 50) healthy volunteers living in urban areas who do not carry out activities related to the field and not occupationally exposed to triazoles, were evaluated. Previous studies used controls with a number of samples similar to the present study, where it was possible to observe statistical differences between the exposed and control groups [9,21]. In this study, differences were also found between both groups, with the need for a larger control group being unnecessary.

Some of the volunteers data collected through the questionnaire (supplementary material) are presented in Table 1. The data show different profiles between volunteers from rural and urban areas in terms of schooling, with a lower level being observed in the rural area group. Statistical differences were not found between groups of smokers and non-smokers for the employed effect bioindicator. Likewise for the variable recent exposure to X-rays (less than 60 days), alcoholic and non-alcoholic ($p > 0.05$ Mann Whitney test).

When observing the average time in years of exposure to triazole fungicides, the group residing in rural areas had an average of 19.4 years, suggesting chronic exposure. To evaluate the influence of the age on the frequency of cytome assay changes from rural residents, the group (n = 145) was stratified into two groups: those over 40 years old (n = 102) and those under 40 years old (n = 43). They were compared them using the Mann-Whitney test with a 5% significance level. The *p*-values for binucleated = 0.3001, karyolitic = 0.7934, karyorrhectic = 0.1657, micronucleus = 0.9800, and pyknotic = 0.7944.

Additionally, the investigation focused on the triazole fungicides used in coffee plantations. Predominantly, the commercial product named OPERA® (epoxiconazole as active ingredient) ranked first, followed by PrioriXtra® (cyproconazole as active ingredient) and Verdadeiro® (cyproconazole as active ingredient).

In order to understand the main forms of exposure to triazole fungicides, information was collected on the mode of application of fungicides in crops. The costal pump was the predominant method, followed by volunteers who reported carrying out the application using a tractor without a protective cabin and both forms of application (tractor without a protective cabin and costal pump), as verified by Ref. [9]; which studied the exposure to complex mixture of pesticides.

To assess the exposure of volunteers to triazole fungicides, the developed analytical method was applied in the urine samples as a bioindicator of exposure and exhibited linearity in 10–200 μg L^{-1} for cyproconazole, metconazole, and triadimenol, and 30 μg L^{-1} to 200 μg L^{-1} for epoxiconazole and propiconazole ($R^2 > 0.98$). Precision and accuracy (variation coefficient <15%) within the parameters suggested by the validation guides consulted [16–18]. The urinary triazoles fungicide was higher in the group exposed occupational and environmentally with an average of 46.21 μg L^{-1}, when compared with which related no occupational exposure, in the rural population. Epoxiconazole was the predominant triazole fungicide found, with 21 positive samples from men and 11 samples from women, both living in rural areas. Followed by 7 samples from men for cyproconazole and 1 sample from man for triadmenol. Urinary triazoles were not detected in any of the samples from the control group.

To identify the cell types in the present study, the scientific literature [9,20], which provides information on the existing cell types and their respective morphologies. Fig. 1 shows the cell types found in volunteers of this study.

After analysis, a comparison was made of each cellular alteration evaluated between the group residing in the rural area and the group residing in the urban area. The mean values, standard deviation, median and *p*-value of each cell change evaluated are shown in Table 2. It is possible to observed that there was a statistical difference ($p < 0,001$) for all cell types, with higher averages of cell changes in the group residing in the rural area when compared with the group residing in the urban area.

In order to better assess the cellular damage presented by the volunteers and the factors that could influence these results, analysis of variance was performed using the Kruskal-Wallis test. Through this analysis, statistical differences were found between men and women living in rural areas, for karyolytic and pyknotic cells, with higher means in the male group. Although there was no statistical difference, higher averages of binucleated cells, karyorrhexis, and cells with micronucleus were observed in the group occupationally/environmentally exposed, as well as the average of urinary triazoles found, suggesting a relationship between cell damage and occupational exposure to triazole fungicides, as shown in Fig. 2.

The data are not influenced by the personal habits, smoking and alcoholism, as presented in Fig. 3.

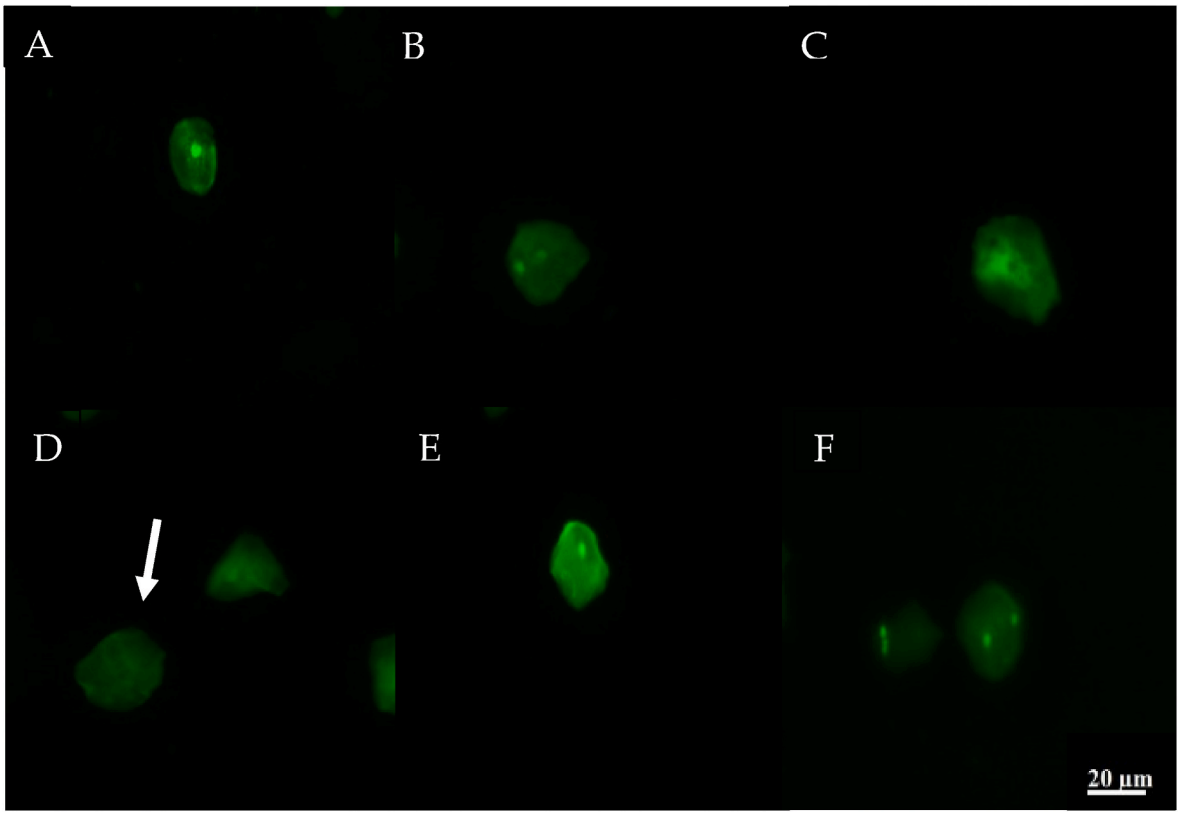

Fig. 1. Photographs of slides of the volunteers of the present study, were different cell types with their respective morphologies were evidenced. From the author. Oral mucosal cells from study volunteers: (A) Normal differentiated cell, (B) Binucleated cell, (C) Cell in karyorrhectic, (D) Karyolytc cell. (E) Pyknotic cell. (F) Cell with micronucleus. 400x magnification.

4. Discussion

The presence of higher concentrations of triazole fungicides in urine samples from volunteers suggests a relationship with occupational exposure. The need for crop productivity facilitates the use of pesticides by farmers, contributing to the risk of exposure. Previous studies found concentrations of triazole fungicides between 10.38 μg L^{-1} to 136.18 μg L^{-1} in samples from farmers [4]. Thus, in this work, an analytical curve from 10 to 200 μg L^{-1} for cyproconazole, metconazole, and triadimenol and 30 μg L^{-1} to 200 μg L^{-1} for epoxiconazole and propiconazole was optimized and validated. The lack of maximum permitted limits for these substances in urine reinforces the importance of further studies being carried out in this area. As previously described by Ref. [4]; the fungicides epoxiconazole and cyproconazole are fungicides commonly used by farmers in the southern region of Minas Gerais, Brazil. In this study, the same classes of pesticides were found through detection in urine samples by LLE - GC-MS, in addition to the data collected through the questionnaire. The detection of triazole fungicides in volunteers who do not apply the pesticide to crops is an indication that not only occupational exposure, but also indirect exposure through the environment and contact with contaminated clothing should be taken into account.

Table 2
Mean, standard deviation and median of cellular alterations found in cells of the oral mucosa of volunteers residing in rural and urban areas.

Parameters	[a]Residents in the rural area (n = 145)	[b]Residents in the urban area (n = 50)	(*p*) Test Mann-Whitney
Binucleated	15.30 ± 82.92 11.50	4.35 ± 95.68 4.00	<0.001
Karyolitic	112.00 ± 114.36 84.50	18.79 ± 52.68 16.00	<0.001
Karyorrhectic	84.62 ± 112.67 75.50	15.07 ± 54.35 12.00	<0.001
Micronucleus	5.26 ± 131.11 3.00	0.075 ± 663.32 0.00	<0.001
Pyknotic	16.47 ± 117.55 10.00	4.75 ± 71.91 4.5	<0.001

Note: The results presented above refer to the arithmetic mean of 2000 analyzed cells, with two analyzes of 1000 cells each.
*p-value obtained through the Mann-Whitney statistical test, with 5% significance.
[a] Rural residents = men and women.
[b] Residents in urban areas = men and women.

Different classes of pesticides are applied by farmers to crops, such as organophosphate insecticides [9]. However, the periods for each application are different. December is a favorable period for the application of triazole fungicides, due to the high rainfall in the region [4]. Furthermore, it is known that triazole fungicides have antagonistic effects on organophosphates, which makes simultaneous application to the crop unfeasible [21]. In agreement, through the applied questionnaire, it was verified that the majority application of triazole fungicides, with variations only in the brand of the commercial product.

The way to evaluate which chemical agent has been absorbed into the body and caused an imbalance is through biomonitoring. Thus, the data here evaluated suggest a correlation between exposure to triazole fungicides (bioindicator of internal dose) and cellular damages (biomarker of effect), since only this class was applied during the sample collection period. Besides the fact that the urinary analysis of triazoles was performed, all the male volunteers in the group residing in the rural area responded that they had come into contact with triazole fungicides, saying that they were responsible for applying the substance and, consequently, may be exposed to the pesticides.

Previous studies have reported pesticide exposures in rural workers in the south of Minas Gerais, Brazil [4,9]. Some limitations may be related to the excessive and often unprotected exposure of rural farmers

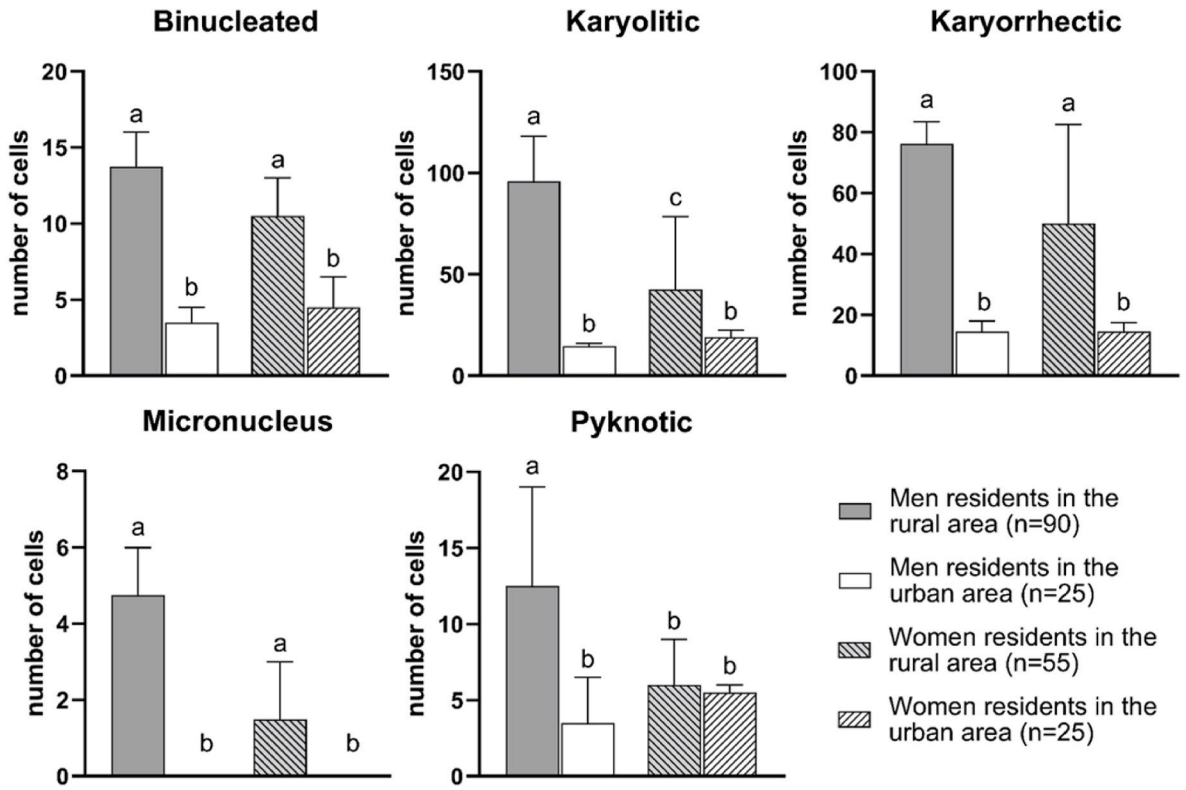

Fig. 2. Frequency of cellular changes observed in the studied groups through analysis of variance. From the author
Note: Frequency values expressed as median and 95% confidence interval. *p-value obtained through the Kruskal-Wallis test, with 5% significance. Values with equal letters did not show significant difference (Dunn's test, 5% significance).

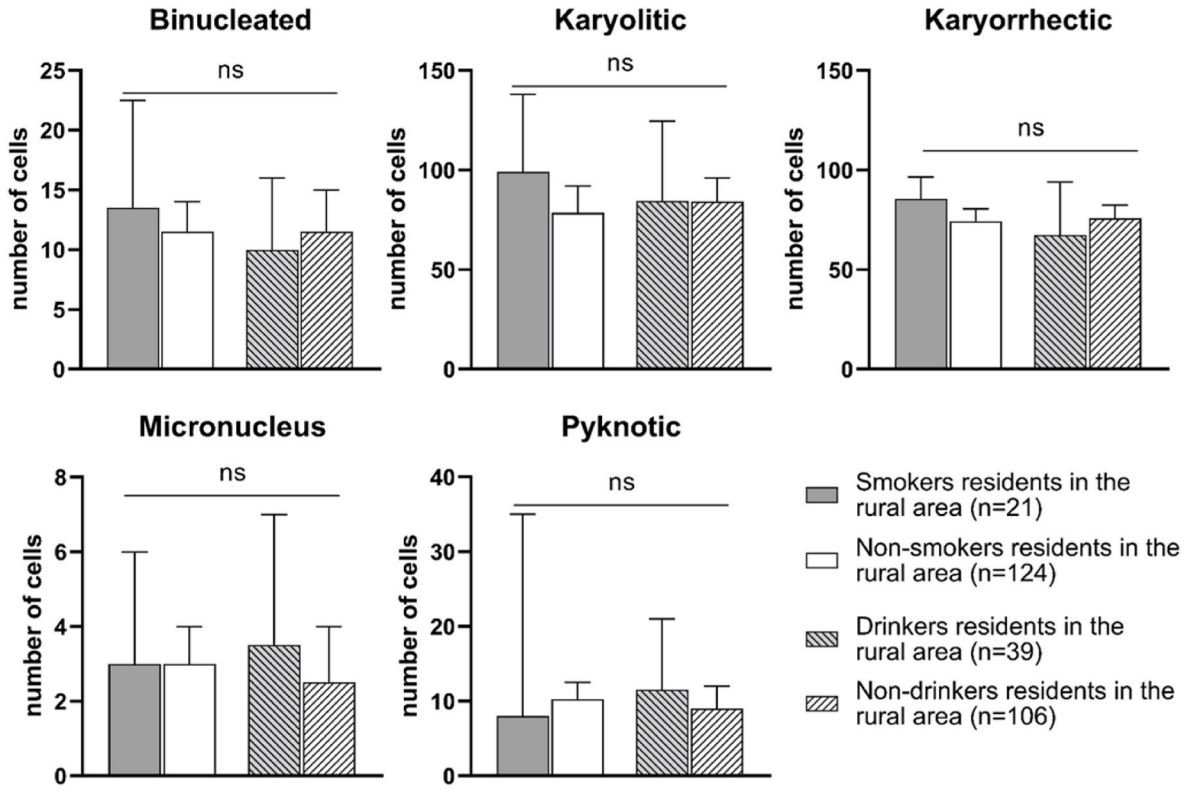

Fig. 3. Frequency of cellular changes observed in the group of rural residents, assessing smoking and alcohol intake, using analysis of variance. From the author
Note: Frequency values expressed as median and 95% confidence interval. *p-value obtained through the Kruskal-Wallis test, with 5% significance.

to pesticides. [22] showed that the low level of schooling, often frequent in rural groups, contributes negatively to the understanding of the correct ways and the importance of using PPE (Personal equipment protection). Bondori et al. (2019) reported that 48% of the volunteers in their survey reported making low or very low use of PPE. Similarly, 49% reported having little or no knowledge of the hazards at the time of pesticide spraying. In this work, it was found that 74,49% of the study population has not completed high school, suggesting that it is one of the contributing factors to exposure, despite a lack of care in the correct way to proceed. All these factors negatively contribute to exposure, increasing the risks of cell damage that can be observed through the BMCyt assay.

As it is possible to observe in Table 2, there is an increase in the cellular damage in the groups residing in rural areas compared with those residing in urban areas. This result suggests that the triazoles exposure is dangerous to this population. The BMCyt assay can be used as a sensitive and early effect indicator, as it is capable of enabling the early diagnosis of possible pathologies to which they may evolve, such as hormonal changes. Thus, being able to provide prior damage to health managers, so that preventive and/or corrective measures are taken, reducing or depriving the volunteer of the source of exposure [9].

In the study by Ref. [23]; the BMCyt technique was used on healthy individuals from different regions of Brazil. Different laboratories carried out the collection of samples from the buccal mucosa, randomizing the slides after preparation and redistributing them to the analysts of the participating laboratories. No statistical differences were observed between the analyzed samples from different regions. In this present study, similar profiles were found among healthy volunteers from the urban area with mean frequency of harmful cells, similar to the study carried out by Ref. [23]. In relation to the group residing in the rural area, there was a greater discrepancy in the means, with greater cell damage observed, which can be explained by exposure to triazole fungicides.

The results are in accordance to those obtained by Ref. [9]; in which the group exposed to organophosphates showed significant changes in all parameters evaluated by the BMCyt assay. These are compared with a control group with many of the same characteristics observed in the present study. In the study of [10]; a statistical difference was found in cells with MN, binucleated, kariolytic, kariorrhectic, and pyknotic in males exposed to pesticides through tea cultivation when compared to the control group without occupational exposure to pesticides.

Many factors can influence the interpretation of the biological indicators, like gender, age, personal habits, general health status, and others. The variability between different populations is a factor that can make it difficult to standardize possible reference values. Thus, it is appropriate to measure the control population with characteristics similar to those of the studied group. In addition, different analysts can present varied results from scored cells samples, which can generate variability and difficulty in establishing reference values [23]. For this reason, the training of the analyst for the correct identification of cell types is indispensable.

The results of this study suggest that women living in rural areas present an environmental exposure to pesticides. Different forms of exposure can cause different cell damage, which is facilitated by intensity, form, and exposure time, among other variables [24]. [24] describe that cellular changes may come from environmental, chemical, and lifestyle factors. Exposure to these agents leads to an increase in oxidative stress, which in turn generates the genetic damage observed through cellular changes. It is noteworthy that cell damage is most often proportional to the intensity of exposure [9,24].

It is known that cells with MN are cells where chromosomes or fragments appear in the cytoplasm of the cell outside the main nucleus. Their formation can occur spontaneously due to chromosomal structural transformations resulting from environmental factors or mitotic failure. Binucleated cells are cells with two central nuclei in the same cytoplasm, and their formation is associated with cell division disorders, such as cytokinesis. Karyorrhectic cells are cells where nuclear fragmentation often occurs as result of apoptosis. Karyolitic are cells where, in this case, the cell nucleus is completely destroyed, which may also be related to the mechanisms of apoptosis, making its microscopic visualization impossible. Pyknotic are cells where, the cell cytoplasm and nucleus are smaller when compared to a normal differentiated cell, which can be caused by the induction of some physical and/or chemical aggressive agent [11,25].

In this study, greater cellular damage was observed in the male group, which is responsible for the application being occupationally exposed. Likewise, the average concentration of triazole fungicides found in this group was higher, which confirms the exposure and suggests a causal relationship between exposure of triazoles fungicides and cellular damage. As can be seen in Fig. 2, there is a statistical difference for karyolytic and pyknotic cells between men and women in rural areas, with higher means for men. These results suggest the existence of a high rate of cell death in this group, which can be caused by apoptosis or necrosis [26], possibly induced by exposure to pesticides. The data presented are not enough to express the causes of the cell death process, however, the existence of cell disturbance in the exposed group, as observed by greater cell damage, suggests greater risks to this population. There is in the body an enzymatic defense system that has the function of controlling oxidative stress [27]. However, when the exposure is very intense, the defense system is not able to contain the stress, culminating in genetic alterations through the mutation of nitrogenous bases in the DNA chain [28]. In association, there is still in the human biological system, a base repair system, that has the function of repairing mutated bases [29]. Similarly, when there is constant exposure to a certain mutagen, this is not able to contain all the mutations, leading to the persistence of genetic damage and the inadequate formation of other cell types [29].

One important variable to be investigated is the possibility of smoking interfering with the genotoxicity results. [9]; found a non-statistical difference in MN among smokers when compared to non-smokers. In this study, no significant differences in cell damage were reported in smokers compared to non-smokers, in alcoholists, or in individuals who did not ingest alcohol (Fig. 3). The statistical analysis with the stratification of the rural group showed that the age variable was not influencing (or biasing) the results of the frequencies of changes in the studied biomarkers. It is known that recent exposure to X-Ray can lead to genetic mutations [29,30]. In this way, the existence of volunteers who were exposed to X-Ray for a period of less than 60 days before the collection of biological samples was evaluated. As the renewal of the oral epithelium lasts about 45 days [20], individuals exposed to X-Ray in older periods were not separated. No significant differences in cell damage were reported in the rural residents group who reported being exposed to X-Ray for less than 60 days compared to the unexposed group. In this research there was no participation of volunteers affected by severe comorbidities, such as neoplasms, in order to avoid possible interference in the results of the BMCyt assay. Thus, the present study narrows the relationship between cell damage observed with exposure to triazoles fungicides since the most common variables were eliminated, either by low sampling, or by statistical insignificance.

5. Conclusions

Therefore, in this study, it was possible to observe the presence of DNA damage, chromosomal instability, and cell death through the BMCyt assay in men living in rural areas who were occupationally and environmentally exposed to triazole fungicides. Consequently, the risk of genotoxic effects induced by exposure to pesticides is higher in this population. Although it is not possible to identify the primary causes of the cell death process, the existence of genotoxic effect observed suggests significant risks for this population. Additionally, the results observed in the group of women are also alarming and demonstrate the urgency of implementing public measures to manage this dangerous scenario.

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Author statement

Luiz F. Costa: methodology, validation, investigation, data curation and writing-review and editing. Luiz P. A. Marciano: methodology, validation, investigation, data curation and writing-review and editing. Fernando Feltrim, Josiane O. Freire, Gislaine B. Silva: investigation. Alessandra C.P. Silvério: project administration, writing-review and editing. Isarita Martins: conceptualization, methodology, supervision, project administration, writing-review and editing, data curation, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2023.110689.

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metabolites

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Article

An Exploratory Study of the Metabolite Profiling from Pesticides Exposed Workers

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Abstract: Pesticides constitute a category of chemical products intended specifically for the control and mitigation of pests. With their constant increase in use, the risk to human health and the environment has increased proportionally due to occupational and environmental exposure to these compounds. The use of these chemicals is associated with several toxic effects related to acute and chronic toxicity, such as infertility, hormonal disorders and cancer. The present work aimed to study the metabolic profile of individuals occupationally exposed to pesticides, using a metabolomics tool to identify potential new biomarkers. Metabolomics analysis was carried out on plasma and urine samples from individuals exposed and non-exposed occupationally, using liquid chromatography coupled with mass spectrometry (UPLC-MS). Non-targeted metabolomics analysis, using principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) or partial least squares discriminant orthogonal analysis (OPLS-DA), demonstrated good separation of the samples and identified 21 discriminating metabolites in plasma and 17 in urine. The analysis of the ROC curve indicated the compounds with the greatest potential for biomarkers. Comprehensive analysis of the metabolic pathways influenced by exposure to pesticides revealed alterations, mainly in lipid and amino acid metabolism. This study indicates that the use of metabolomics provides important information about complex biological responses.

Keywords: pesticides; untargeted metabolomic; UPLC-Q-TOF-MS; occupational toxicology; plasma; urine

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1. Introduction

Pesticides constitute a heterogeneous category of chemicals designed specifically for the control of pests, weeds or plant diseases. The human population is environmentally and occupationally exposed to various pesticides, whose applications have increased significantly in recent years. Despite being necessary for pest control, they represent a serious problem for human health and the environment, mainly due to the lack of control and inspection of exposure. Several health and environmental risks are associated with these chemicals, which can lead to acute or chronic toxic effects, including infertility, hormonal disorders, psychiatric disorders and cancer. For these reasons, pesticides have been the object of studies, both for the damage they cause to human health as well as for the damage to the environment and the emergence of resistance in organisms [1–6].

Considering the potential risk to human health, biomonitoring is a useful tool to assess the risk of workers exposed to pesticides. Usually, the determination of the activity of cholinesterase enzymes is used for this purpose, but in many cases, this determination has

been shown to be insufficient to characterize the harmful effects resulting from exposure to different pesticides or mixtures of them. In addition, this biomarker is ineffective for the early indication of deleterious effects induced by toxic agents [7–10]. In this sense, characterizing the metabolic response to pesticides and how they influence human health becomes a very important step, and metabolomics presents itself as a useful alternative capable of achieving this goal.

Metabolomics has been used in several areas to understand cell functioning and biological changes in organisms. This technique provides an integrated view of biochemistry in complex systems and, in the area of occupational and environmental toxicology, is especially useful in the identification of new biomarkers of chemical contaminants, in addition to exhibiting great potential for clarifying the mechanisms of toxicity of these agents as well as investigating metabolism and other possible biological interactions. There are some challenges involved with metabolomics, such as the analytical process, which is critical, in addition to difficulties involved with reproducing the data necessary for the replication of studies or even their continuity after the discovery of biomarkers [11–14].

Based on metabolite coverage, there are different analytical approaches to metabolomics, classified as targeted analysis and untargeted analysis. Non-targeted metabolomics has been successfully applied in environmental and occupational toxicology studies, providing relevant information on the mechanisms of toxic action and even identifying biomarkers [15–20].

Due to the complexity of biological systems and the physicochemical diversity of all metabolites, it is necessary to use different analytical techniques; the main ones are nuclear magnetic resonance—NMR—and mass spectrometry—MS—coupled with different techniques of separation, such as liquid chromatography, gas chromatography or capillary electrophoresis. Liquid chromatography is currently recognized as the most used technique in global metabolomics [11,21–23]. However, there are few studies on the elucidation of the metabolic profile in individuals exposed to pesticides.

Thus, the objective of the present study was to carry out an exploratory experimental analysis to identify whether there are differences in the plasmatic and urinary metabolic profile of individuals exposed and not exposed occupationally to pesticides, using ultra-performance liquid chromatography coupled with an ionization mass spectrometer by quadrupole electrospray with time of flight (UHPLC-ESI-Q-TOF-MS). Multivariate statistical analysis was used to evaluate the data and identify discriminating metabolites between the two groups, with the purpose of providing important information about complex biological responses and discovering possible candidates for biomarkers that can be used in clinical practice to promote biomonitoring.

2. Materials and Methods

2.1. Population and Sample Collection

The study population consisted of 40 male individuals, aged between 20 and 62 years, who were divided into two groups. The exposed group consisted of 20 rural workers from the city of Paraguaçu, Minas Gerais, Brazil, who were occupationally exposed to pesticides. The control group consisted of 20 male volunteers, living in Belo Horizonte and region, Minas Gerais, Brazil, with no history of occupational exposure to xenobiotics.

All volunteers who participated in this study signed a free and informed consent form before collecting the biological material. Subsequently, 10 mL of peripheral blood was collected by venipuncture and 50 mL of urine was collected in sterile bottles, in an average period of 7 days after pesticide application. All samples were immediately transported, in a refrigerated box, to the laboratory, where they were stored at −70 °C until analysis.

2.2. Ethical Aspects of Research

This study was submitted and approved by the ethics and research committee of the Federal University of Minas Gerais—UFMG (CAAE: 39339720.0.0000.5149, opinion number: 5.473.586).

2.3. Standards and Reagents

The chromatographic-grade solvents methanol, formic acid, 2-propanol, acetonitrile and sodium formate were obtained from Sigma-Aldrich® (San Luis, MO, USA).

2.4. Sample Preparation

2.4.1. Plasma Preparation

Plasma samples were frozen at −70 °C to promote metabolic quenching. To perform the analysis, the samples were thawed and vortexed; then a 100 µL aliquot of plasma was extracted, using 300 µL of a chloroform: methanol (2:1, *v*/*v*) mixture. The samples were vortexed for 30 s and centrifuged at 14,000× *g* for 10 min at 4 °C, and then 5 µL of the supernatant was injected into the UPLC-QTOF-MS. Quality-control samples (QCs) were prepared by mixing 20 µL of each plasma sample and processed in the same way. Sample blanks were also prepared, containing all reagents used in the preparation except for plasma [24,25].

2.4.2. Urine Preparation

Urine samples were thawed and vortexed for 30 s. Then, 100 µL was pipetted into 125 µL of methanol in Eppendorf microtubes and vortexed again for 30 s. Samples were centrifuged at 14,000× *g* for 10 min at 4 °C for protein precipitation. In sequence, 200 µL of the supernatant was transferred to properly labeled flasks and 300 µL of an aqueous solution containing 0.1% methanoic acid was added. Quality-control samples (QCs) were prepared by mixing 20 µL of each urine sample and processed in the same way. Sample blank preparations were also performed, where all reagents except plasma were pipetted [25].

2.5. Analytical System

After preparation, plasma and urine samples were analyzed by ultra-performance liquid chromatography (Shimadzu®, Tokyo, Japan) coupled with a mass spectrometer (Bruker®, Billerica, MA, USA). For chromatographic separation, a C_{18} reversed-phase column (100 mm × 2.1 mm 1.8 µm particle) was used, and temperature was maintained at 40 °C. A linear gradient elution of solvent consisting of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid was performed as follows: 98–2% A (0–8 min), 9010% A (8–14 min), 50–50% A (14–14.50 min), 0–100% A (14.50–16.50 min) and 98–2% A (16.50–20 min) at a flow rate of 0.4 mL/min. More details are described in Table S1. The injection volume was 5 µL for all samples, and they were analyzed under the same analytical conditions, randomly, to avoid uncertainties and artefacts related to the injection order and to prevent the effect of gradual changes in instrument sensitivity over entire batches.

The mass spectrometer was operated in positive mode, using electrospray ionization (ESI) and collision energies of 20 and 50 eV (each 50% of the time). The source conditions were set to nebulizer gas (N_2) at 5.0 bar, drying gas (N_2) at 9.0 L/min, dry gas temperature at 200 °C, capillary voltage at 4.5 KV; the ionization source was set to 4500 V. A solution of hexakis (2,2-difluoroethoxy) phosphazene in 2-propanol was used for lock mass calibration, and a sodium formate solution was used for calibration before analysis. Ions in the range of 20 to 1000 m/z were monitored, with an acquisition rate of 4 Hz. In order to monitor the stability and reproducibility of the analytical conditions, 3 blank solvents and 5 QC samples were injected at the beginning of the sample test, another 3 blank solvents were injected at the end of the test and 1 QC sample was injected every 5 study samples. Data were acquired using Hystar Application® version 3.2 and OtofControl® software version 3.4 (Bruker Daltonics Corporation®, Billerica, MA, USA).

2.6. Detection and Identification of Non-Target Metabolites

Data files obtained by UPLC-MS were converted to mzML format using the Proteowizard® software (http://proteowizard.sourceforge.net/download.html (accessed on 24 September 2022)). The statistical software R® version 4.2.1 and the XCMS Bioconductor® package version 3.12 (http://bioconductor.org/packages/release/bioc/html/xcms.html (accessed on

24 September 2022)) were used for pre-treatment of the data, including baseline correction, denoising, deconvolution, peak alignment and generating the data matrix. The analysis of the results was performed using the centWave algorithm in XCMS®, with snthresh = 5.0, mzwid = 0.01 and bw = 5. Urine-related data were normalized by the corresponding urinary creatinine concentration, and plasma sample results were normalized by the median of intensities. Data filtering was applied to all data to remove features with relative standard deviation (RSD) higher than 30% from the subsequent analysis. After that, logarithmic transformation was used as well as scaling by mean centering. This processing was performed using the MetaboAnalyst® software version 5.0 (https://www.metaboanalyst.ca/ (accessed on 24 September 2022)).

The putative identification of the metabolites recognized by UPLC-QTOF-MS was performed by searching for m/z's in public databases, such as the Human Metabolome Database (HMDB®) and Metlin®, in addition to the CEU Mass Mediator platform—where a search is carried out in different databases simultaneously (Kegg, HMDB®, LipidMaps®, Metlin®)—considering a maximum mass error of 5 ppm and the adducts $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$.

2.7. Statistical Analysis

The plasma and urine results obtained were subjected to multivariate statistical analysis using unsupervised chemometric methods, such as principal component analysis (PCA), and supervised methods, such as partial least squares discriminant analysis (PLS-DA) and orthogonal discriminant analysis by partial least squares (OPLS-DA). Cross-validation was used to validate supervised models. All these analyses were performed in MetaboAnalyst® 5.0. From the PLS-DA and OPLS-DA models, discriminating molecular features between the groups were identified using the variable importance in projection (VIP) and volcano plot classification, adopting the criteria of VIP > 1.5; FDR < 0.01, *p*-values between analyzed groups < 0.05 and fold change > 2.0.

Descriptive statistics were also applied to evaluate numerical variables, such as mean, standard deviation and median. The Shapiro–Wilk and Anderson–Darling tests were used to verify whether the variables had a Gaussian normal distribution. Categorical variables were evaluated in terms of frequency and percentage. To verify the hypotheses of association between variables with normal distribution, the t-test was used; for the others, the Mann–Whitney test and Fisher's exact test were used. An association was considered statistically significant when the *p*-value was less than 0.05. The software used in the analyses was Graphpad Prism® version 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA, 2020).

3. Results and Discussion

3.1. Population Characteristics

The main characteristics and distribution in relation to the study population are presented in Table 1. The mean age of individuals in the control group was 39 years, ranging from 20 to 62 years, and the mean age of the exposed group was 45 years, ranging from 22 to 62 years. Most volunteers in this group (70%) worked with family agriculture on small and medium-sized properties, while 75% of the control group had an administrative function. All participants in the exposed group had been in direct contact with pesticides for more than three years, with 44% of them reporting more than 20 years of exposure.

Comparisons made between the exposed and control groups for the variables schooling (time of study), age group and occupation showed that only for the variable occupation was there a significant association ($p < 0.001$), indicating that there is a higher prevalence of individuals with administrative function among individuals in the control group, while among those exposed, the highest prevalence was for family agriculture and applicator.

Table 1. Comparison of variables between exposed and control groups.

Variables	Exposed (n = 20)	Control (n = 20)	p-Value
Age group [2]	n (%)	n (%)	
20 a 30	2 (10.0)	8 (40.0)	
31 a 40	6 (30.0)	3 (15.0)	0.118
41 a 55	9 (45.0)	5 (25.0)	
>55	3 (15.0)	4 (20.0)	
Scholarity (Years) [1]	5 (4–8)	16 (12–20)	0.223
Occupation [2]			
Administrative	0 (0)	15 (75)	
Family agriculture	14 (70)	0 (0)	<0.001 *
Applicator	6 (30)	0 (0)	
Student	0 (0)	3 (15)	
Other	0 (0)	2 (10)	

Note: [1] Mann–Whitney test; [2] Fisher's exact test; * p-value < 0.05.

3.2. Identification of Plasma Metabolic Profile of Individuals Occupationally Exposed to Pesticides

After the processing steps, multivariate statistical methods were applied to the data matrix. These were initially evaluated using the unsupervised pattern recognition method principal component analysis (PCA) with quality control (QC) prediction, to find similarities and differences between the samples and verify the presence of trends or outliers. Figure 1 shows a good separation of samples from individuals occupationally and non-occupationally exposed to pesticides, reflecting differences in their metabolic profiles. PCA analysis was used to assess the quality of data acquisition, and the strong clustering of QC samples, observed in the center of the graph (Figure 1), indicated analytical stability, quality and reliability of the data obtained.

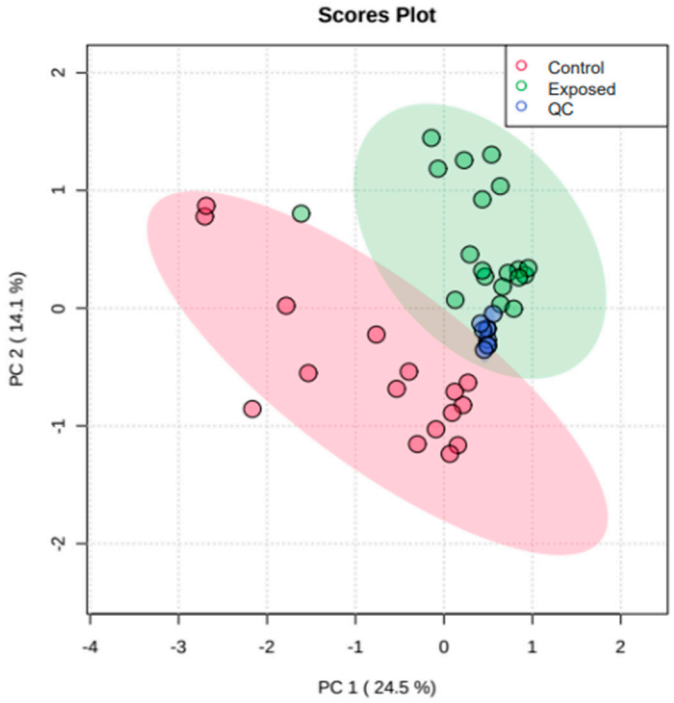
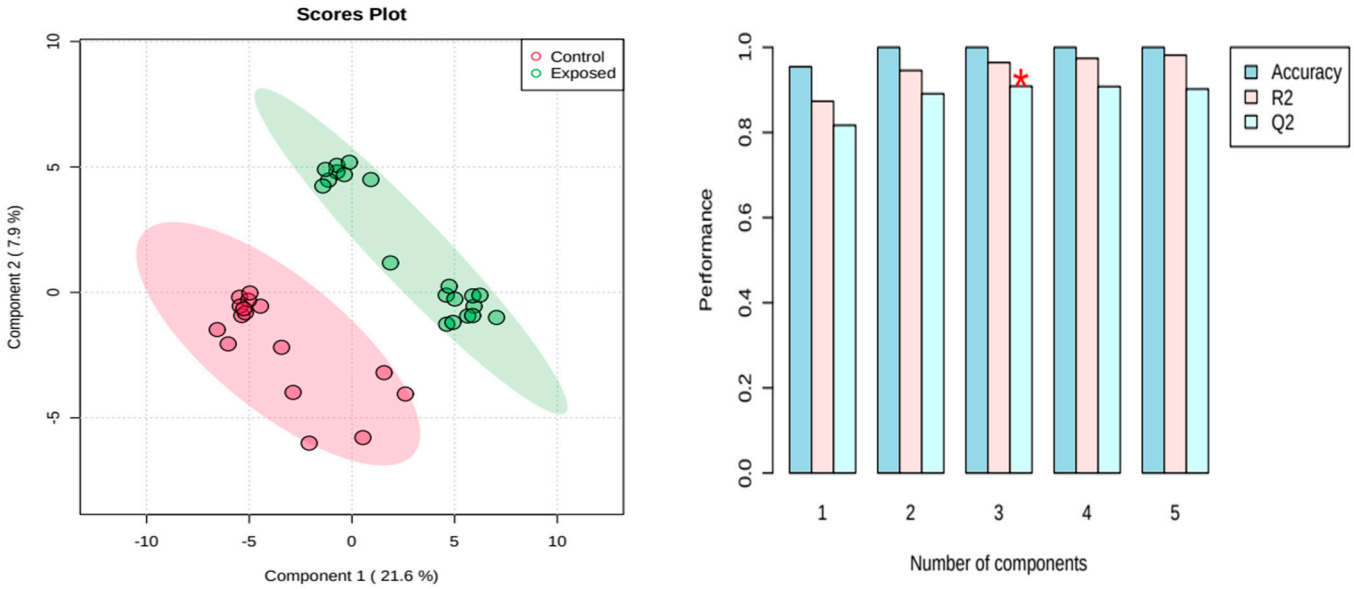


Figure 1. Score graph of principal component analysis (PCA) models for plasma samples from individuals exposed and non-exposed occupationally to pesticides as well as quality control samples (QCs).

The results were then evaluated by the supervised pattern recognition method discriminant partial least squares analysis (PLS-DA) to model the differences between the metabolites responsible for the separation of groups. The score graph (Figure 2) shows an effective separation between the plasma samples from the control group versus the exposed group. In order to test the validity of the constructed model, a cross-validation test was carried out, which can be seen in Figure 2B. The cross-validation results were $R^2 = 0.98$, $Q^2 = 0.92$, which shows an adequate fit of the data to the model, and therefore it is considered a highly accurate model. Usual values for biological experiments are $Q^2 > 0.4$ and $R^2 > 0.7$ [26,27].



(**A**) (**B**)

Figure 2. Partial least squares discriminant analysis (PLS-DA) indicating effective discrimination between the group of occupationally exposed individuals and the control group (volunteers not occupationally exposed to pesticides) (**A**) and PLS-DA classification by cross-validation, using different number of components. The red star indicates the best classifier (**B**).

To maximize the separation between groups and find discriminating metabolites, the supervised partial orthogonal least squares discriminant analysis (OPLS-DA) classification method was also used. The score graph obtained is shown in Figure S1.

Using volcano plot analysis, which allows selection of significant features based on biological significance and statistical significance, differential metabolites were identified, based on criteria of fold change > 2.0, FDR < 0.01, VIP > 1.5 and *p*-value < 0.05. The VIP value, obtained in the OPLS-DA model, indicates the importance of each metabolite in the discrimination between groups. Thus, using all these criteria, 14 molecular features were found with lower and 11 with higher results when comparing the control group with the exposed group, which is represented in Figure 3.

Based on the results obtained, 25 molecular features that contributed most to the discrimination between groups were selected. The identification of four of these features was not possible due to non-recognition of the respective m/z by the databases used. The results of this evaluation and the possible identification are compiled in Table 2, where the VIP score, *p*-value, fold change and chemical classification of the compounds are also presented.

To evaluate the performance of these compounds as potential biomarkers in the evaluation of toxicity induced by pesticides, the ROC curve (receiver operating characteristic) was used, which is a graphic representation that illustrates the performance of a compound related to its discriminatory power. An area under the curve (AUC) of

0.5 indicates that a substance has no discriminating ability, while an AUC of 1.0 represents a test with perfect discrimination. AUC values > 0.85 are considered acceptable for clinical applications [28]. Based on these results, 16 candidates performed satisfactorily, including 1-[2-chloro-4-(4-chlorophenoxy)phenyl]-2-(1,2,4-triazol-1-yl)ethanol (AUC = 1.0), PG(18:3(9Z,12Z,15Z)/22:4(7Z,10Z,13Z,16Z)), with AUC = 0.998; Ceramide (AUC = 0.99) and Sphingomyelin (AUC = 0.90). On the other hand, five compounds, 5-O-b-D-glucopyranoside, 13,14-Dihydro PGE1, Val-Gly-Asp, phosphatidylethanolamine and phosphatidylcholine, had AUC < 0.85. The ROC curves of some possible biomarkers are shown in Figure S2.

According to the ROC curve results, the most discriminating compound between the groups was 1-[2-chloro-4-(4-chlorophenoxy)phenyl]-2-(1,2,4-triazol-1-yl)ethanol (AUC = 1), a metabolite of difenoconazole, a fungicide of triazole class widely used by the workers involved in this study, according to their reports.

According to the literature, triazoles act not only on the target enzymes of the CYP51 family (lanosterol-14α-demethylase), necessary for the biosynthesis of ergosterol, but also on other enzymes of the cytochrome P450 family. Exposure to triazole fungicides can trigger endocrine disruption due to inhibition of aromatase activity, oxidative stress, cell apopthesis and inflammatory reactions. A product of CYP51 demethylation in humans is cholesterol, which is required for the synthesis of bile acids, mineralocorticoids, glucocorticoids and sex steroids [19,29].

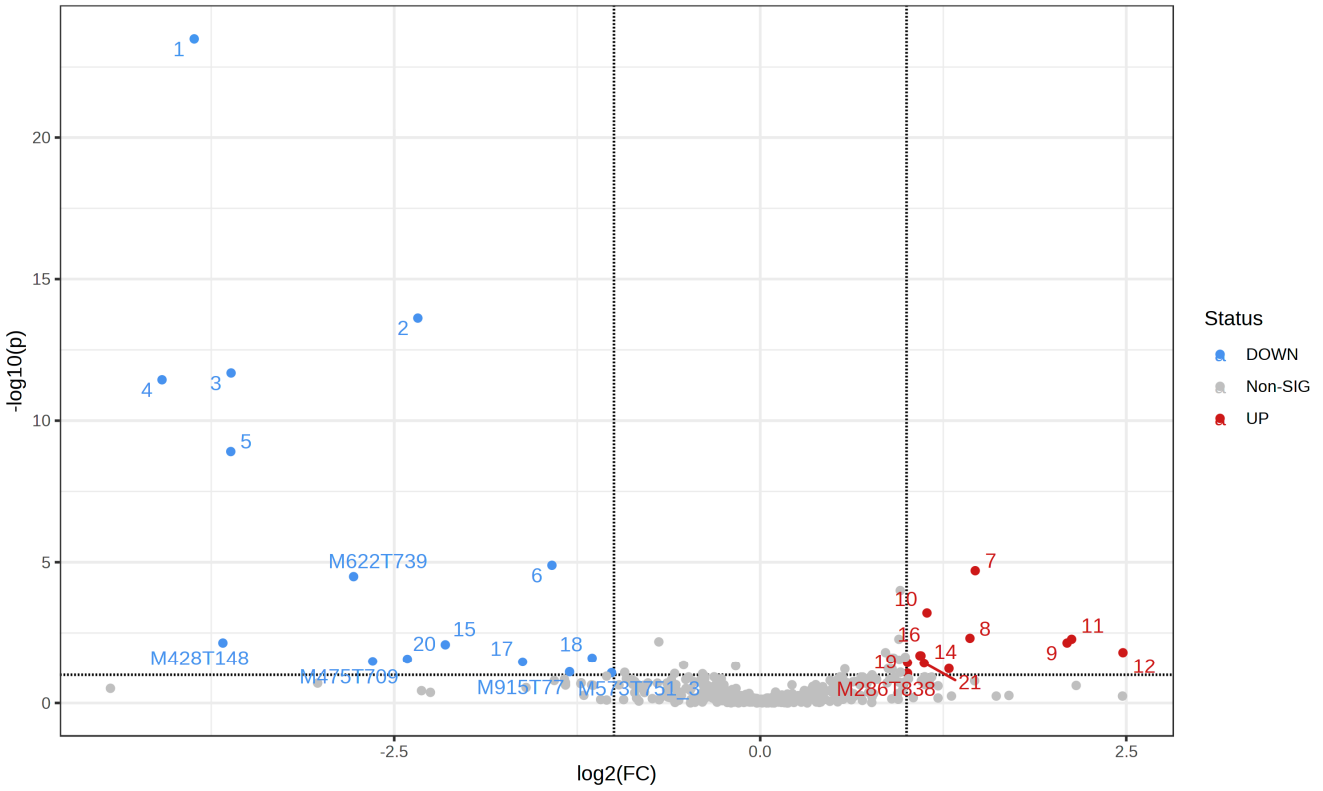


Figure 3. Volcano plot applied to data from plasma samples of individuals exposed and not exposed occupationally to pesticides. Note: each point on the plot represents a molecular feature. The red dots indicate an increase in features in the control group and the blue dots, a decrease in the same group. The gray dots represent a lack of distinction between the groups. The dots identified by numbers correspond to the compounds listed in Table 2.

Table 2. Discriminating compounds identified in the metabolomic analysis using the UPLC-MS technique, in the plasma of individuals exposed and non-exposed occupationally to pesticides.

Identification	Compounds	m/z	[a] VIP Score	[b] Fold Change	[c] *p*-Value	Chemical Classification
1	1-[2-chloro-4-(4-chlorophenoxy)phenyl]-2-(1,2,4-triazol-1-yl)ethanol	349.038	3.50	−3.8528	3.6×10^{-24}	Triazole
2	13-bromo(...)hydroxy-tridecatrienoic acid	389.076	3.39	−2.3291	2.5×10^{-14}	Prenol Lipids
3	PI(20:5(5Z,8Z,11Z,14Z,17Z)/0:0)	618.280	3.36	−3.620	1.9×10^{-12}	Glycerophosphoinositol
4	Diphosphatidylglycerol	709.794	3.31	−4.0838	3.6×10^{-12}	Phosphatidylglycerol
5	Cer(d16:1/LTE4)	692.479	3.17	−3.6229	1.2×10^{-11}	Ceramides
6	Glutamate	147.053	2.57	−1.4101	1.2×10^{-5}	Amino acids, peptides and analogues
7	PG(18:3(9Z,12Z,15Z)/22:4(7Z,10Z,13Z,16Z))	821.071	2.50	1.4805	1.5×10^{-5}	Glycerophospholipids
8	Paraxantine	180.064	2.33	1.4462	3.6×10^{-3}	Purine and derivatives
9	5-aminophthalazine-1,4-diol	194.083	2.31	2.1139	6.3×10^{-3}	Benzodiazine
10	LysoPA(0:0/18:1(9Z))	436.258	2.15	1.1522	4.8×10^{-4}	Glycerophospholipids
11	Sphingomyelin	730.598	2.01	2.14	4.3×10^{-3}	Sphingolipids
12	1,22-Docosanedioic acid	370.308	2.00	2.4958	1.3×10^{-2}	Fatty acids
13	3-(Methylthio)propanoyl-CoA	869.689	1.95	1.003	1.8×10^{-2}	Glycerophospholipids
14	5-O-b-D- glucopyranoside	903.255	1.94	1.1131	1.6×10^{-2}	Carbohydrates
15	13,14-Dihidro PGE1	356.256	1.93	−2.138	7.8×10^{-3}	Lipids
16	Val Gly Asp	289.127	1.90	1.1063	1.6×10^{-2}	Amino acids, peptides and analogues
17	Phosphatidylethanolamine (18:3)	475.269	1.79	−2.402	3.4×10^{-2}	Phosphatidylethanolamine
18	Phosphatidylcholine (20:0/14:0)	762.092	1.78	−1.1386	2.3×10^{-2}	Phosphatidylcholine
19	Norepinephrine sulfate	249.030	1.77	1.13	3.3×10^{-2}	Arylsulfate
20	Phosphatidylcholine (14:1)	465.285	1.75	−2.6305	2.5×10^{-2}	Phosphatidylcholine
21	L-tirosine	180.073	1.64	1.1367	3.4×10^{-2}	Amino acids, peptides and analogues

Note: [a] VIP score was obtained from the OPLS-DA model; [b] negative fold change values correspond to increased compounds in the exposed group, while positive values correspond to decreased compounds in this same group; [c] *p*-values were calculated from the nonparametric Mann–Whitney test between groups exposed and non-exposed occupationally to pesticides.

Jiang et al. [30] and Teng et al. [29] used transcriptomics and metabolomics by LC-MS/MS to verify the toxicity of difenoconazole in zebrafish. The analysis of metabolic pathways, after exposure to this pesticide, detected changes in amino acid metabolism, including glutamate, lipid metabolism, energy metabolism and nucleotide metabolism, among others. Both authors stated that fungicides disrupted lipid metabolism at biochemical, transcriptomic and metabolomic levels and concluded that the two techniques, when used together, may be useful to verify the toxicological effects of difenoconazole in zebrafish and to assess the risks of xenobiotics in aquatic organisms.

An interesting evaluation was performed by Van Meter et al. [31] on the influence of exposure to pesticide mixtures on the metabolomic profile of frogs. They tested three herbicides, an insecticide and a triazole fungicide, exposing the amphibians to a single compound and mixtures of these. They observed that the metabolites and pathways impacted were different between treatments, indicating that the modes of action of xenobiotics may change depending on chemical interactions with other toxicants. However, the authors reported that regardless of the mode of action, some metabolites commonly altered by these mixtures were amino acids and lipids, which are critical for protein synthesis, DNA structure and replication and as a response to oxidative stress. Similar results were observed in our study.

3.3. Analysis of Metabolic Pathways Affected by Exposure to Pesticides Based on Plasma Metabolomics Results by UPLC-QTOF-MS

After identifying the discriminating metabolites, we sought to understand the metabolic pathways involved in the biological response generated by exposure to pesticides. For this, the selected variables were subjected to metabolic pathway analysis, which was conducted using the MetaboAnalyst® software (https://www.metaboanalyst.ca/ (accessed on 24 September 2022). The results are represented in Figure 4, which shows all corresponding pathways according to pathway impact values and *p*-values from the pathway enrichment analysis. The size of the circle represents the influencing factor, and the color identifies the importance of the pathway for understanding the biological response. Therefore, the larger and red circles are considered the most important pathways, i.e., the most disturbed ones.

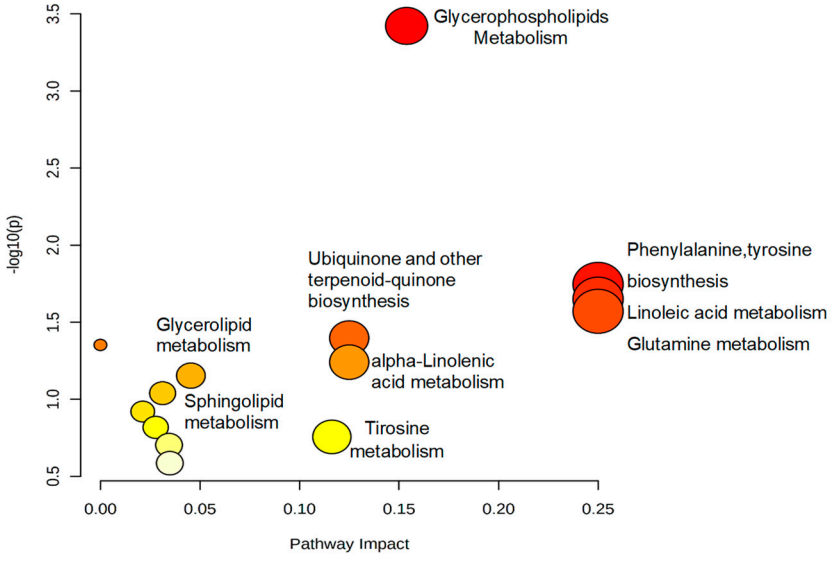


Figure 4. Representation of the metabolic pathways involved in the biological response associated with exposure to pesticides. The colors, varying from yellow to red, represent metabolites with different levels of significance.

The alterations were found in fourteen metabolic pathways, as shown in Figure 4; the main ones were glycerophospholipid metabolism, phenylalanine/tyrosine biosynthesis, linoleic acid metabolism and glutamine metabolism.

3.3.1. Metabolism of Glycerophospholipids and Linoleic Acid

Lipids are organic compounds that can be divided into eight categories, according to the classification proposed by LIPIDMAPS (http://www.lipidmaps.org (accessed on 26 November 2022)): fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenollipids, saccharolipids and polyketides. Studies in the literature report that this chemical group plays an important role in living systems due to its biological functions and effects on human health, such as energy storage, cell membrane structure, cell communication, regulation of biological processes and relationship with cardiovascular and other chronic diseases [14,18].

In this study, alterations in the classes of glycerolipids, sphingolipids and fatty acids were identified and corroborated by studies found in the literature.

Wang et al. [32] and Weng et al. [33] studied the toxicity of epoxiconazole triazole exposure in zebrafish, and both reported that triazoles can induce an imbalance of oxidative homeostasis, causing disturbances in energy metabolism, lipid metabolism and amino acid metabolism. Yang and colleagues [34] explored the association between maternal exposure to 37 pesticides from different classes and gestation length in rats. By metabolomic analysis, the authors realized that the most affected pathway was glycerolipid metabolism and concluded that these positive changes were related to exposure to pesticides.

Nguyen et al. [14] evaluated the neurotoxicity of the fungicides strobilurin, azoxystrobin and trifloxystrobin in human neuroblastoma cells. They stated that the concentration of some lipids was increased when cells were exposed to these toxicants when compared to the control group, such as phosphatidylcholine, phosphatidylethanolamine, triglycerides, PI (14:1_16:0), ceramides and carnitines, among others. The authors said that these compounds are essential components in neuronal and mitochondrial cells, and the altered expression of these lipids may be related to mitochondrial dysfunction.

Similar results were revealed by the metabolomics of this study, such as increased levels of phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine.

The structural diversity of these glycerophospholipids plays a key role in membrane fluidity and stability. Studies report that due to the increased demand for membrane constituents, there is an increase in phosphatidylcholine synthesis in cancer cells and solid tumors. Its reduction has been observed in pathological conditions in the liver in humans, including liver failure. Regarding phosphatidylglycerol, high concentrations of this substance were detected in acute coronary syndrome and may be linked to the pathogenesis of cardiovascular diseases. As for phosphatidylethanolamine, research claims that these compounds may be related to vascular diseases and increased incidence of cancer, in addition to playing an important role in other diseases [35–37].

Wang et al. [38] performed untargeted integrated lipidomics and metabolomics analyses to verify the effects of imidacloprid and acetamiprid pesticides on neuronal cells. Pathway analysis demonstrated that glycerophospholipid and sphingolipid metabolism were the most affected. According to the authors, changes in the concentration of glycerophospholipids indicate changes in the composition and permeability of the cell membrane and that the increase in phosphatidylcholine found may have occurred due to disruption of the composition of the cytoskeleton and glycerophospholipids caused by the entry of xenobiotics into cells, resulting in alterations in the composition and content of phosphatidylcholines. On the other hand, exposure to imidacloprid suppressed fatty acid synthesis.

Sanchez et al. [19] studied the neurotoxicity of triazoles, propiconazole and tebuconazole in mitochondrial dysfunction and alteration of lipid metabolism in human cells. According to the authors, neurotoxicity can occur through several mechanisms, such as oxidative stress and free radical formation, mitochondrial impairment and apoptosis. In the study conducted by these authors, it was proven that triazoles act on these mechanisms and, as a biological response, lipid alterations occur. They found altered levels of phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol, in addition to identifying new lipids that may be significant in neurodegenerative diseases.

Another altered metabolic pathway identified in this study was the metabolism of 9-12-octadecadienoic acid (linoleic acid) and 9-12-15-octadecatrienoic acid (α-linolenic acid). These lipids, called polyunsaturated fatty acids, play an important role in maintaining the membrane, signal transduction and anti-inflammatory properties, in addition to participating in the synthesis of hemoglobin and cell division. They are responsible for modulating transmission in the cholinergic, serotonergic and dopaminergic systems and are involved in neurotrophic support and oxidative stress by modulating the expression of genes responsible for these actions [39,40].

The most important metabolite of linoleic acid in animal tissues is arachidonic acid, and several families of eicosanoids are derived from this acid, including prostaglandins, thromboxanes and leukotrienes. In this study, plasma metabolomics showed an increase in prostaglandin E1 (PGE1) in occupationally exposed individuals.

Prostaglandins are prostanoids synthesized from arachidonic acid by cyclooxygenase enzymes (COX-1 and COX-2). They have essential homeostatic functions in renal physiology, are potent vasodilators and platelet aggregation inhibitors but are also implicated in many pathological conditions, such as inflammation, cardiovascular disease and the initiation of carcinogenesis, representing the link between inflammation and cancer. Under normal conditions, levels of prostanoids in cells are low, but during a disturbance, their concentration can be altered [13,35].

In a study carried out by Yan et al. [41], the serum metabolic profile of individuals exposed to pesticides was evaluated by LC-MS. Among the results described by the authors, changes were observed in pathways related to inflammation, including metabolism of arachidonic acid and prostaglandins, which were associated with exposure to xenobiotics. According to the authors, oxidative stress can increase the production of arachidonic acid, an inflammatory intermediate that can be converted into prostaglandins. Pabst et al. [42] performed lipidomics in patients with acute myeloid leukemia and reported changes in a large number of plasma lipids, including prostanoids. The authors showed that arachidonic acid precursors and their metabolites are positively related to disease severity and prognosis.

Tyurina et al. [43] performed an LC/MS analysis of cardiolipins in plasma from insecticide-exposed rats. Among the results found by the authors, increased levels of arachidonic acid and linoleic acid were detected and correlated with neuroinflammation and oxidative stress, causing neurodegeneration in the animals. They cited that these findings may contribute to a better understanding of the pathogenesis of Parkinson's disease and lead to the development of new biomarkers of mitochondrial dysfunction.

Another study was developed by Birch and collaborators [44], who investigated the effects of endocrine disruption of pesticides on calcium influx in human spermatozoa. They found that these toxicants can interfere with human sperm function through effects on the Ca^{2+} channel induced, for example, by increased PGE1, causing deficiencies in capacitation, sperm motility and chemotaxis towards the ovum. According to the authors, the findings were able to relate the effects of exposure to pesticides with human fertility.

The metabolism of sphingolipids was another pathway highlighted as important in exposure to pesticides in this study. Sphingomyelin showed a lower concentration in the workers' plasma compared to the control group, whereas ceramide was present at more concentrated levels.

Sphingolipids are physiologically related to the regulation of cell growth, differentiation, and apoptosis. Among the types of sphingolipids, ceramides are structural components of the membrane and secondary messengers in cell signaling [19,45].

Results similar to ours were found by Weng et al. [33] when studying the toxicity of exposure to triazole epoxiconazole in zebrafish. The authors believe that the activation of enzymes responsible for the conversion of sphingomyelin into ceramide (sphingomyelinases), caused by exposure, explained the decrease in sphingolipid.

Pabst et al. [42] and Robinson et al. [46] studied the relationship between reactive oxygen species (ROS) and the metabolome in acute myeloid leukemia cells. The authors

detected lower levels of sphingolipids in the plasma of these individuals and, after an overall analysis of the findings, concluded that ROS are important in regulating the synthesis and/or degradation of sphingolipids.

As evidenced in the literature, the lipid group is involved in several biological processes, such as oxidative stress, inflammation, obesity and endocrine disruption. Many xenobiotics exert their toxic effects on these biological processes and, therefore, the investigation of changes in lipid metabolism becomes an important strategy to elucidate mechanisms of toxic action, in addition to the possibility of identifying new biomarkers of exposure to these chemical substances [45,47,48].

3.3.2. Phenylalanine/Tyrosine Biosynthesis and Glutamine Metabolism

Amino acids are the building blocks of our body in the form of proteins and protein complexes, and many important metabolites, such as neurotransmitters, purines and pyrimidines, among others, are products of cellular amino acid metabolism [49].

In this study, some amino acids were altered in the exposed group compared to the control group. For example, tyrosine had reduced levels, while glutamate had increased levels.

The change in relation to tyrosine is important, as it is responsible for numerous functions in the body, being a precursor of several neurotransmitters such as dopamine, noradrenaline, adrenaline and thyroid hormones. This amino acid is synthesized from the hydroxylation of phenylalanine by the enzyme phenylalanine hydroxylase. According to studies reported in the literature, deficient tyrosine biosynthesis can lead to the accumulation of phenylalanine in body fluids and also to a reduction in the production of catecholamines (adrenaline, noradrenaline and dopamine). These concentration changes may be related to the occurrence of neurodegenerative and neuropsychological symptoms, such as changes in motor activity, attention deficit and hyperactivity [49,50]. Thus, its decrease could promote these effects in the population exposed to pesticides.

Gao et al. [49] studied the physiological and biochemical changes caused in larvae exposed to the insecticide Spinetoram by transcriptomic and metabolomic analyses. Among the alterations found in the metabolism of amino acids, the authors cited a decrease in tyrosine in the studied group, which consequently generated a decrease in dopamine, causing Parkinson's disease in the exposed insects. Rodrigues et al. [51] also detected tyrosine levels reduced by exposure to pesticides in neuronal cells and correlated changes in amino acid levels with neurodegenerative conditions in patients with Alzheimer's disease.

Ch et al. [52] analyzed the metabolic profile of the saliva and urine of male farmers exposed to pesticides. They found results similar to our study, such as changes in amino acid levels, and stated that oxidative stress due to exposure to xenobiotics caused disturbances in amino acid and energy metabolism.

Yan et al. [41] verified the hypothesis that exposure to neonicotinoid insecticides causes disorders of amino acid metabolism, lipid accumulation and oxidative stress in mice. By evaluating non-target metabolomics, they observed significant increases in some amino acids, such as phenylalanine, suggesting that tyrosine biosynthesis was disturbed. Furthermore, they claimed that the increase in branched-chain amino acids and phenylalanine also induced lipid accumulation, which was consistent with the increase in lipid compounds identified by them in animals exposed to neonicotinoids. This exposure also increased levels of glutamate, which is associated with increased energy needs. These results are in line with those found in this study.

Regarding glutamine, this amino acid is involved in the synthesis of nucleic acids, nucleotides and proteins, among others. Its metabolism involves two enzymes: glutamine synthase, which is related to the synthesis of glutamine, and glutaminase, which acts in its conversion into glutamate, the latter being an important amino acid in cell metabolism that can be converted into gamma-aminobutyric acid (GABA), glucose, urea, synthesis of other amino acids or glutathione. Studies claim that negatively regulated glutamate uptake can contribute to the accumulation of glutamate in the synaptic cleft, causing a neurodegenerative process called excitotoxicity [51,53].

Changes induced by exposure to pesticides in the brain metabolome were studied by Rodrigues et al. [51]. In this review, they reported that deviations were observed in the level of metabolites related to several metabolic pathways, including energy metabolism, mitochondrial dysfunction, lipid and amino acid metabolism. There was an increase in glutamine and glutamate levels in the brain, affecting the pathway that is considered the main regulator of glutamate levels. Wang et al. [32] and Bonvallot et al. [54] also found similar results in zebrafish exposed to epoxiconazole and in a group of pregnant rats exposed to a mixture of pesticides, respectively.

Cattani et al. [55] investigated the effects of glyphosate exposure on some neurochemical and behavioral parameters in rats. Their results showed that exposure to this pesticide caused oxidative stress, in addition to affecting cholinergic and glutaminergic neurotransmission in the hippocampus of the animals. The authors concluded that neurotoxicity induced by glyphosate after exposure involves the phenomenon of glutamate excitotoxicity, due to increased release of glutamate in the synaptic cleft, lower uptake of this by interaction of glyphosate with receptors, leading to increased ionic flow of Ca^{2+} to the hippocampus cells. These events culminate in oxidative stress, astrocytic dysfunction and depressive behavior.

3.3.3. Ubiquinone Biosynthesis

Ubiquinone, or Coenzyme Q, is a ubiquitous lipid that is involved in electron transport and oxidative phosphorylation. The biosynthesis of this compound is a multienzymatic process involving several precursors and, in mammals, the group attached to ubiquinone is derived from the essential amino acid phenylalanine, which is converted to tyrosine and, later, 4-hydroxybenzoate [6,56,57]. In this study, the metabolic alteration of this pathway was evidenced by the decrease in tyrosine in the workers' plasma.

Payet et al. [56] evaluated the initial steps in the ubiquinone biosynthesis pathway in yeast. The authors combined techniques such as isotopic labeling, chemical analogue supplementation and genetics to identify enzymes associated with the tyrosine metabolic pathway and stated that the increase in tyrosine in cell culture raised the level of ubiquinone, concluding that this amino acid is used as a precursor of Coenzyme Q and that their concentrations are directly proportional.

A study developed by Vujić et al. [57] verified toxicity signature pathways in human brain endothelial cells exposed to the herbicide Paraquat. The analysis of the metabolic pathways highlighted the metabolism of ubiquinone as the most significant pathway. According to the authors, the results suggest that the studied herbicide modulates mechanisms such as oxidative stress and pathways related to hypoxia in endothelial cells. They also mentioned that Paraquat is an inducer of oxidative stress that inhibits the complexes of the respiratory chain in the mitochondria, especially complex I, which is directly related to the metabolism of ubiquinone.

Park et al. [58] studied the intracellular metabolomic alteration of the insecticide carbofuran, using non-targeted metabolomics by LC-MS. They observed alterations in the metabolism of amino acids, nucleosides (purine and pyrimidine) and ubiquinone biosynthesis. The metabolic pathways related to 4-hydroxybenzoate production and ubiquinone biosynthesis showed significant differences between the two groups studied, and the authors concluded that these changes were probably related to the response to oxidative stress induced by carbofuran. Considering the results of the study, the authors highlighted the importance of non-targeted metabolomics as a good strategy to identify intracellular alterations after exposure to xenobiotics.

The metabolomic analysis of the plasma of this study population demonstrated that several metabolites were significantly modulated by exposure to pesticides. The observed metabolic changes are consistent with studies found in the literature and may be associated with impaired membrane function, oxidative stress, inflammation, mitochondrial dysfunction and endocrine disruption.

3.4. Identification of Urinary Metabolic Profile of Individuals Occupationally Exposed to Pesticides

Urine samples were prepared according to the sample preparation methodology previously described and were analyzed under the same analytical conditions, randomly, with QC samples interspersed every 5 urine samples. After the processing step (deconvolution, grouping and alignment), the data were normalized by urinary creatinine, a recommended procedure to correct the effect of variable dilutions in specific samples [59]. In sequence, they were subjected to multivariate analysis techniques. Initially, principal component analysis (PCA) was applied to all samples, including the QCs, to assess a possible separation between groups of individuals occupationally and non-occupationally exposed to pesticides. The developed model is represented in Figure 5 and shows a weak separation between the groups, but a rigorous grouping of the QCs revealed analytical stability and good quality of the acquired data.

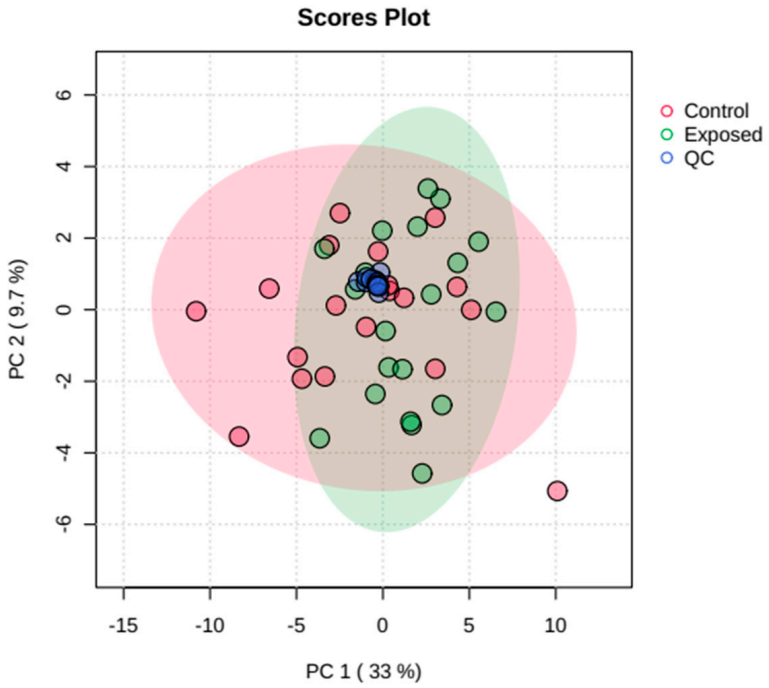


Figure 5. PCA model of urine samples from workers occupationally and non-occupationally exposed to pesticides as well as quality control samples (QCs).

Subsequently, data were analyzed using the supervised multivariate PLS-DA method. Figure 6 shows that there was a better separation between the groups.

The constructed PLS-DA model presented a good performance, with variation explained by the model (R^2) of 0.99 and predictive capacity (Q^2) of 0.68, for five principal components. As previously mentioned, $R^2 > 0.7$ and $Q^2 > 0.4$ are acceptable for biological experiments [27].

Then, the OPLS-DA model was applied, and the result is shown in Figure S3. Using this model, the constructed graphs provide better visualization of the separation between samples, but in this study, few discriminating metabolites with significant values were found (correlation and covariance > 0.8). Therefore, we decided to use the VIP values obtained in the PLS-DA analysis.

The criteria used for analysis of urine samples by LC-MS were the same as applied to plasma samples (VIP > 1.5, FDR < 0,01, *p*-value < 0.05 and fold change >2.0).

Based on the analysis carried out by volcano plot, 05 molecular features were found with lower results and 12 with higher results when comparing the control group in relation to the exposed one, as shown in Figure 7.

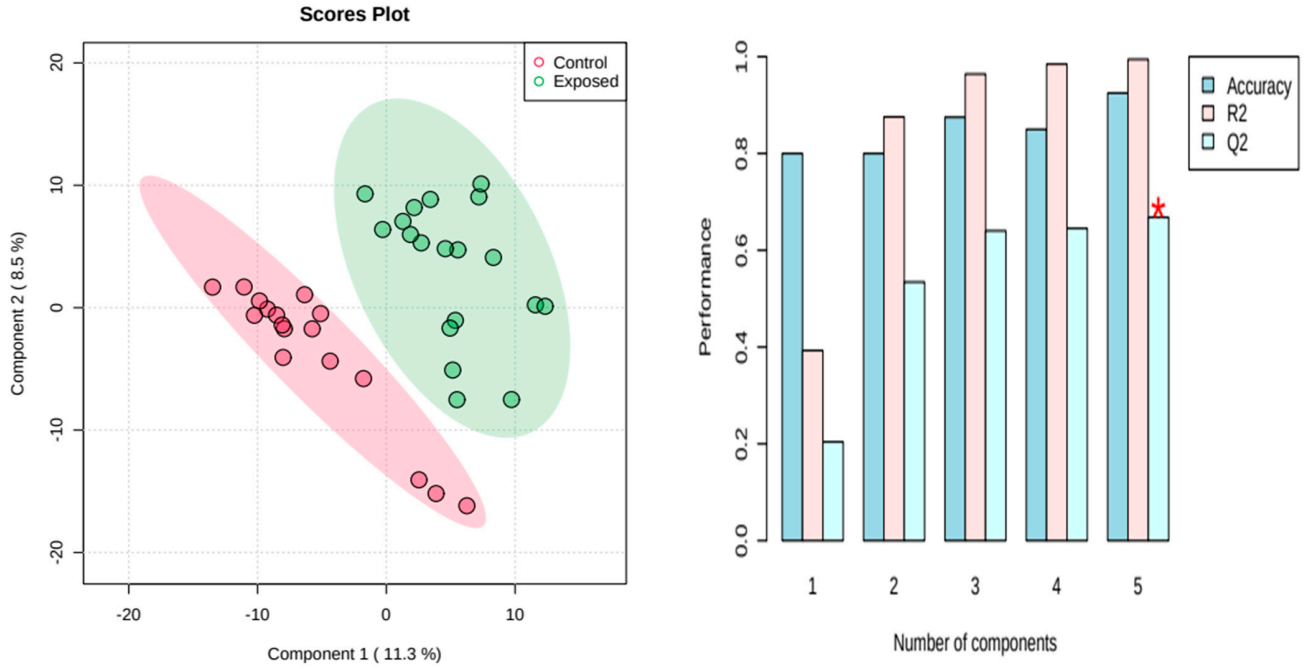


Figure 6. (**A**) PLS-DA model developed for urine samples from volunteers in this study, analyzed by LC-MS. (**B**) Validation of the PLS-DA model by cross-validation, using different number of components, with quality parameters R^2 = 0.99 (variation explained by the model) and Q^2 = 0.68 (model predictability). The red star indicates the best classifier.

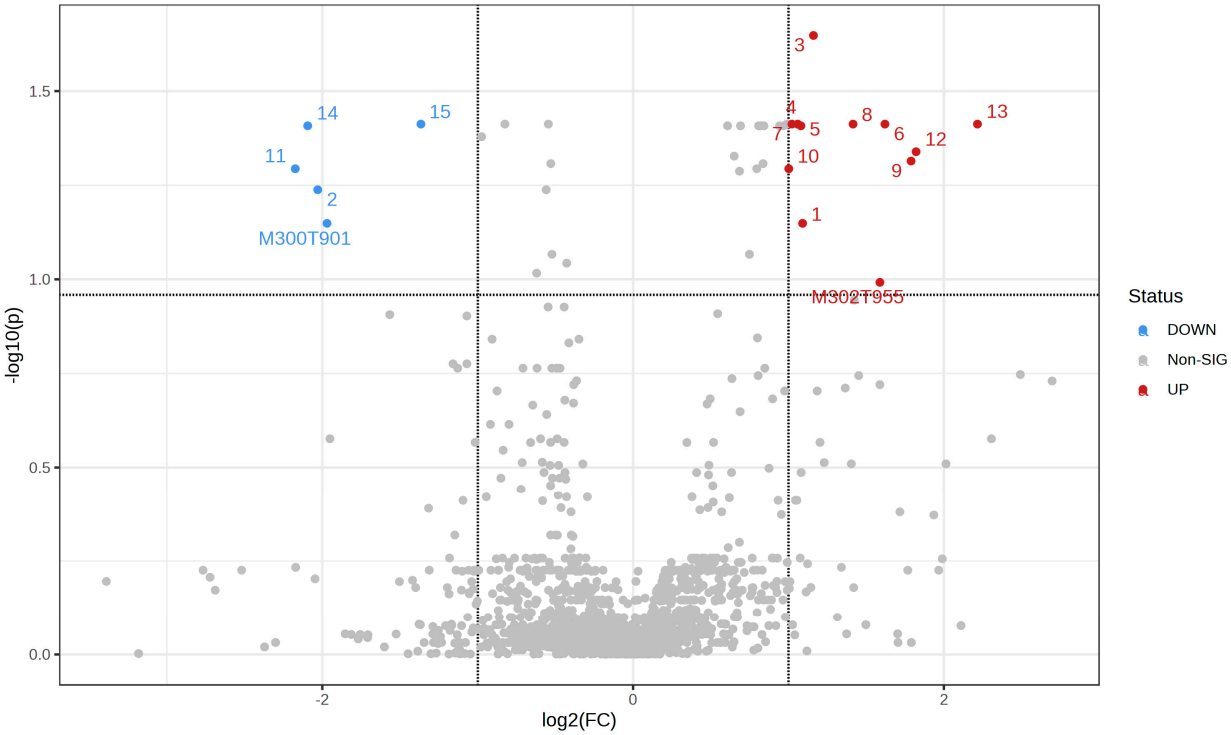


Figure 7. Volcano plot applied to data from urine samples of individuals occupationally exposed and not exposed to pesticides. Note: each point on the plot represents a molecular feature. The red dots indicate an increase in these and the blue dots, a decrease in them. The gray dots represent a lack of distinction between the groups. The dots identified by numbers correspond to the compounds listed in Table 3.

The identity of these metabolites was then searched on HMDB®, Metlin® and Lipidmaps® databases, considering a maximum mass error of 5 ppm. Two molecular features were not identified by the databases. Table 3 shows the data compiled from this evaluation and the possible identification of substances.

Table 3. Discriminating compounds identified by UPLC-MS in the metabolomic analysis of urine samples from workers occupationally exposed and not exposed to pesticides.

Identification	Compounds	m/z	[a] VIP Score	[b] Fold Change	[c] *p*-Value	Chemical Classification
1	Phosphoribosylamine	229.035	4.4742	2.8165	3.0×10^{-2}	Pentose phosphate
2	Carbendazim	191,187	3.2886	−1.4924	6.0×10^{-3}	Benzimidazole
3	Triacylglycerol	852.757	3.2706	1.1967	2.9×10^{-4}	Glycerolipids
4	1-Palmityl-2-palmitoleoyl-glycero-3-phosphocholine	717.567	3.2557	1.1233	3.8×10^{-3}	Glycerolipids
5	PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z) /18:4(6Z,9Z,12Z,15Z))	783.483	3.2477	1.1553	3.9×10^{-3}	Glycerolipids
6	N-Acetylgalactosamine	221.208	3.2183	1.637	4.5×10^{-3}	Carbohydrates
7	TG(10:0/10:0/10:0)	919.767	3.0001	1.0771	1.0×10^{-3}	Glycerolipids
8	SM(d18:1/12:0)	646.504	2.9576	1.0084	2.0×10^{-3}	Sphingolipids
9	UDP-D-galacturonic acid	581.034	2.9366	1.0137	2.3×10^{-3}	Pyrimidine nucleotide
10	TG(15:0/18:0/O-18:0)	834.804	29.296	1.0899	2.0×10^{-2}	Glycerolipids
11	Glycosyl 2-{6-(2-cyanophenoxy)pyrimidine-4-yloxy}benzoate	495.127	2.9051	−1.291	2.0×10^{-2}	Benzenoid
12	Diphosphoinositol tetraphosphate	819.794	2.797	1.0012	4.5×10^{-3}	Inositol phosphate
13	PE(18:3(6Z,9Z,12Z)/P-18:0)	725.535	2.7486	1.0137	1.0×10^{-3}	Glycerolipids
14	Mycophenolic Acid Glucuronide	496.158	2.4476	−1.276	3.4×10^{-3}	Carbohydrates
15	Histidine	155.069	2.139	−1.1325	3.1×10^{-3}	Amino acids, peptides and analogues

Note: [a] VIP score was obtained from the PLS-DA model; [b] negative fold change values correspond to increased compounds in the exposed group, while positive values correspond to decreased metabolites in this same group; [c] *p*-values were calculated from nonparametric Mann–Whitney test between groups exposed and non-exposed occupationally to pesticides.

In order to verify the performance of selected metabolites as possible biomarkers, the ROC curve analysis was applied, and the results of some compounds are shown in Figure S4.

Based on these results, only the compounds glycosyl-2-{6-(2-cyanophenoxy) pyrimidine-4-yloxy} benzoate and carbendazim showed AUC > 0.85. The first compound (AUC = 0.88) is a metabolite of azoxystrobin, a fungicide also described in the list of substances used by our exposed group. Carbendazim, despite not being on the list of applied products, showed high levels in workers.

Azoxystrobin and other substances of the strobilurin class inhibit mitochondrial respiration by blocking electron transport. They bind to the quinol binding site of the cytochrome b-c1 complex, where ubiquinone (Coenzyme Q10) would normally bind when transporting electrons to this protein. Several animal studies have reported that this compound has the potential for developmental toxicity and neurotoxicity and may be associated with autism, brain aging, neurodegeneration, apoptosis and oxidative stress [60,61].

Mesnage et al. [62] investigated metabolic disturbances in rats by UPLC-MS caused by a mixture of six pesticides, including azoxystrobin, to obtain information on toxicity mechanisms that could act as early biochemical markers of chronic harmful effects. The authors found increased levels of some amino acids in the group exposed to the mixture of xenobiotics, in addition to decreased levels of glycerolipids, results that are in line with our findings. They concluded that there was oxidative stress resulting from exposure to the mixture of pesticides. In addition, they claimed that transcriptomic and metabolomic

approaches to risk assessment procedures can result in greater sensitivity, accuracy and predictability of results, with positive implications for public health.

Bauer et al. [63] performed a UPLC-QTOF-MS screening to identify and characterize metabolites of thiacloprid, azoxystrobin and difenoconazole pesticides in plant crops and food. They addressed the degradation pathways of these xenobiotics during a kinetic study, in addition to the degradation of the original compounds. Among the results obtained, one of the metabolites detected was glycosyl 2-{6-(2-cyanophenoxy) pyrimidine-4-yloxy} benzoate. The authors mentioned that the metabolites found in the study are generally not detected in routine analyses, as these normally include only predefined metabolites and active compounds. They declared that the developed method provided new and important information about the presence and distribution of compounds related to the metabolism of xenobiotics.

Another compound that showed discriminant performance was carbendazim (AUC = 0.86), a broad-spectrum fungicide which has systemic activity of inhibiting the formation of mitotic microtubules during mitosis, affecting the growth and division of spores. According to the literature, this compound is known to manifest embryotoxicity, germ cell apoptosis, teratogenesis and infertility in different species of mammals. It is considered a mutagenic, carcinogenic and toxic agent for development and reproduction [64,65].

Chen et al. [66] used UPLC-MS metabolomic analysis to understand the effects of carbendazim on bee brain metabolism. The authors found, among the positively regulated compounds, carbendazim as one of the most abundant. In addition, they detected glycerolipids with decreased levels in bees exposed to the xenobiotic. According to the authors, the affected metabolic pathways included changes in amino acid metabolism, lipid metabolism, energy metabolism and ubiquinone biosynthesis. These results are in line with those found in our study, where carbendazim was detected at high levels and glycerophospholipids at reduced concentrations in samples from the exposed group, in addition to showing alterations in similar metabolic pathways.

Yang et al. [67] studied the risks of exposure to chlorothalonil, carbendazim, prochloraz and their mixtures in embryonic and larval zebrafish based on metabolomic analysis by LC-MS. The authors detected 26 altered metabolites, which were mainly associated with glycolysis pathways, amino acids and lipid metabolism. According to the authors, amino acids and glucose play important roles in the embryonic development of zebrafish, so the metabolomic analysis provided some important information for understanding the presumed mechanism of the three fungicides studied in aquatic organisms.

Despite the unsatisfactory performance of the other metabolites as possible discriminating compounds, evidenced by the ROC curve analysis, they are important to understand the metabolic disturbances induced by exposure to pesticides.

3.5. Analysis of Metabolic Pathways Affected by Exposure to Pesticides Based on Urine Metabolomics Results by UPLC-QTOF-MS

To understand the metabolic pathways involved in the biological response generated by exposure to pesticides, the variables identified and responsible for discriminating between groups were selected and then subjected to metabolic pathway analysis using the MetaboAnalyst® software.

All corresponding pathways according to the impact values and the *p*-values from the pathway enrichment analysis are plotted in Figure 8. Larger red circles are considered the most influenced pathways.

As shown in Figure 8, alterations were found in six metabolic pathways, and the main changes were in glycosylphosphatidylinositol-anchor biosynthesis, histidine metabolism, sphingolipid metabolism, glycerophospholipid metabolism and purine metabolism.

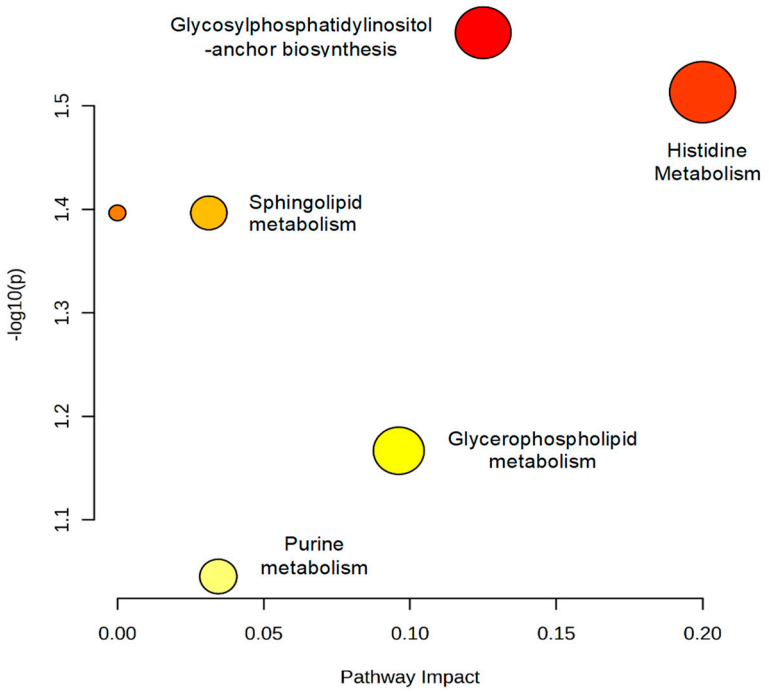


Figure 8. Representation of the metabolic pathways involved in the biological response associated with exposure to pesticides. The colors, varying from yellow to red, represent metabolites with different levels of significance.

3.5.1. Glycosylphosphatidylinositol-Anchor Biosynthesis

Glycosylphosphatidylinositols (GPI) are glycophospholipid structures that act as membrane anchors for many cell surface proteins and are essential for cell viability. The central structure is formed by a lipid group, an inositol group, glucosamine and phosphoethanolamine. The entire structure is anchored to the cell surface by the insertion of phosphatidylinositol fatty acid chains into the membrane bilayer. GPI-anchored proteins and glycoproteins play an essential role in many biological processes, such as cell recognition, activation and interaction, cell surface enzymatic reaction, embryogenesis, fertilization and bacterial and viral infection [68–70].

According to the literature, glycosylphosphatidylinositols can be cleaved by specific phospholipases and the protein can be released. When GPI biosynthesis is defective, these enzymes may not function properly. Studies on the regulation of phospholipid synthesis have focused on the regulation of phosphatidylinositol and phosphatidylcholine synthesis by regulating structural genes in response to the lipid precursors inositol and choline. Variants in specific genes are responsible for defects in glycosylphosphatidylinositol biosynthesis and are associated with broad clinical features, including developmental delay, intellectual disability, seizures and various congenital anomalies [70,71]. In the present study, carried out on the urine of individuals occupationally exposed and not exposed to pesticides, glycerolipids, in general, were reduced in the exposed group in relation to the control group.

Szewczyk et al. [20] used metabolomic studies to understand the influence of xenobiotics on a fungal organism through exposure to the herbicide atrazine. They observed that there was an increase in the perturbation of the membrane, causing greater membrane fluidity, probably due to a decrease in phosphatidylethanolamines. In addition, the authors mentioned that phospholipids containing inositol served as precursors for the synthesis of phosphoinositides and inositol polyphosphates and were involved in the anchoring process of plasmatic membrane proteins. They believe that a reduced level of inositol indicates lower cell viability. With the analysis of all the results obtained, they revealed that the

presence of atrazine in the fungal culture induced oxidative stress, disturbances of amino acid and lipid metabolism and caused an increase in membrane fluidity.

Bernat et al. [72] verified the response of a fungal strain to the herbicide 2,4-dichlorophenoxyacetic acid, regarding the metabolome, membrane fluidity and oxidative stress. The authors observed that in the presence of the toxic compound, there were increases of up to 3 times in the permeability of the membrane when compared to the control group, indicating a significant influence of the compound in this region, and explained that the membrane can be a potential target for the action of this xenobiotic due to its lipophilicity. With this study, the authors demonstrated that the herbicide altered the general concentrations of amino acids and the profiles of fatty acids and lipids, in addition to disturbing the homeostasis of the fungal cell membrane.

The metabolic pathways of sphingolipids and glycerolipids, classes already contextualized in Section 3.3.1, were identified as being negatively altered in the urine samples of individuals occupationally exposed to pesticides.

3.5.2. Histidine Metabolism

Histidine is an amino acid that has several roles in cellular function. It is involved in the biosynthesis of purinergic bases, plays a structural and catalytic role in many enzymes and has important anti-inflammatory, antioxidant and anti-secretory functions in the body. Histidine, by the action of the enzyme histidine decarboxylase, is converted into histamine, a potent mediator of numerous physiological reactions. Histamine is considered a neurotransmitter with different functions in various disorders of the central nervous system, including insomnia, Parkinson's disease, schizophrenia, Alzheimer's disease and cerebral ischemia [73,74].

Liu et al. [75] studied metabolic disorders in mice that were exposed to residues of common pesticides in the diet (chlorfenapyr and acetamiprid), using global metabolomics. They observed that the exposed group presented, among other changes, decreased levels of histamine, resulting in the accumulation of histidine in the body. With the analysis of all results, they concluded that exposure to xenobiotics, even at low concentrations, causes significant changes in the metabolic profile of individuals and that pesticide residues in the diet cause underestimated influences on body health.

In the study carried out by Yan et al. [76], the metabolic profile of individuals exposed to pesticides was evaluated by LC-MS. They found disturbances in metabolic pathways related to oxidative stress, inflammation, lipid and fatty acid metabolism, mitochondrial energy metabolism and neurotransmitter precursors. Among the results described, changes in histidine metabolism were observed, and the authors associated these changes with inflammation and oxidative stress. They claimed that xenobiotics may exert influences on inflammation-related pathways as well.

3.5.3. Purine Metabolism

Among the metabolic pathways influenced by exposure to pesticides, the metabolism of purines was also pointed out, where lower levels of phosphoribosylamine were found in individuals in the exposed group.

Purines are nitrogenous bases that, in addition to being used in the synthesis of DNA and RNA, are important components of several biomolecules, such as ATP, GTP, cAMP, NADH and Coenzyme A. According to the literature, purine biosynthesis requires ten enzymatic transformations to generate inosine monophosphate, and the first step involves phosphoribosylamine, formed from the conversion of 5-phosphoribosyl pyrophosphate by amido-phosphoribosyl-transferase. Phosphorribosylamine is a carbohydrate derivative belonging to the class of organic compounds known as pentose phosphates. The dysregulation of purine biosynthesis has been associated with cancer, gout, neuropathologies and immunological disorders [77,78]. According to McCune et al. [79], the inosine monophosphate generated in the synthesis of purines contributes to the production of several intermediates, such as AMP, GMP, adenosine and inosine. The decrease in its

production leads to a negative feedback inhibition of 5-phosphoribosyl pyrophosphate and prevents the activation, for example, of T-cells, reducing the action of the immune system.

Zhang et al. [80] developed an untargeted metabolomic method to investigate the mechanism of enantioselective toxicity of the insecticide dinotefuran in bees. They observed that the most disturbed pathway was the synthesis/metabolism of purines, associated with energy supply, and deepened the study of the effects of this toxicant on purine-related metabolites. They found 17 up-regulated and 11 down-regulated compounds in the dinotefuran-treated group. Among the metabolites with reduced levels were guanosine monophosphate and inosine monophosphate, indicating alterations in the first stages of purine synthesis. Analyzing all the results obtained in the study, it was indicated that the greatest toxicity of the pesticide was related to the disturbance of the metabolic pathway of purines and its inhibitory role in energy metabolism, concluding that this xenobiotic can endanger the existence of bees by interrupting energetic metabolism.

Kislitskaya et al. [81] analyzed disorders of antioxidant enzymes and purine metabolism in the ejaculate of men exposed to pesticides and air pollutants. The authors claimed that this exposure disturbs the balance of lipid peroxidation and antioxidant activity, activating the formation of free radicals in male germ cells, which leads to increased levels of oxidative stress and decreased purine metabolism. They concluded that these changes may interfere with morphological differentiation and sperm movement.

In general, analyzing all the results of the pathway analysis of this study, we can infer that exposure to pesticides produces toxicity through multiple mechanisms, mainly through oxidative stress, inflammatory reactions and mitochondrial dysfunction.

According to the information provided by the participants of the occupationally exposed group through a questionnaire, important alterations in organic systems were observed. Disturbances have been observed mainly in the central nervous system and peripheral nervous system, where 55% of participants reported symptoms such as headache, muscle weakness and tremors, dizziness and tingling. Alterations in the digestive system were also pointed out, with 50% of workers reporting nausea, heartburn/burning, vomiting, abdominal cramps and diarrhea. Arrhythmia, hypertension, difficulty breathing, nasal irritation and fatigue were identified in 20% of occupationally exposed individuals. It can be seen that the affected pathways identified by metabolomics are directly related to these effects reported by rural workers.

These findings corroborate the toxic effects arising from exposure to pesticides, according to results found by a systematic review carried out by Lopes and Albuquerque [82], where data from 116 studies were gathered that demonstrated the negative impact of exposure to these xenobiotics on human health and the environment. Among the most common symptoms reported by participants were headaches, nausea and stomach pain, dysuria, gastritis, abdominal cramps, respiratory diseases, anxiety, myalgia, irritability and depression.

In this study, we verified the metabolic profiles in plasma and urine samples of individuals occupationally exposed and not exposed to pesticides. It is important to emphasize that compared to plasma, the metabolic profile of urine represents the result of glomerular plasma filtration, reabsorption and tubular excretion; therefore, metabolite concentrations may be different. It should also be noted that this study has limitations, including the small sample size (n = 40), which may have been a source of random variation in the results of the 2 groups analyzed. Furthermore, the identification of the metabolites was putative: Level 2 as defined by the Metabolomics Standards Initiative (MSI).

4. Conclusions

The metabolomic study using the UPLC-Q-TOF-MS technique revealed metabolic disturbances in workers exposed occupationally to pesticides. The disturbances identified involved several metabolic pathways, with emphasis on the metabolism of lipids and amino acids. The multivariate analyses carried out using pattern recognition methods led to the identification of important metabolites for discrimination between the group exposed and

not exposed occupationally to pesticides, 21 of which were plasmatic and 15 of which were urinary metabolites. With the analysis of the area under the curve (AUC), calculated using the ROC curve, the compounds with the greatest potential for biomarkers were revealed. Thus, it can be inferred that the metabolomic analysis contributed to the indication of possible candidates for biomarkers that may be capable of predicting damage to workers' health at an early stage. However, for clinical use, the possible biomarkers need to be validated, using a larger number of individuals per group and following stricter protocols.

So far, there are no published works on the study of metabolites identified by the global metabolomics approach in plasma and urine of human beings exposed to pesticides. For this reason, the results of this study may be of great relevance for understanding the mechanisms of the toxic action of pesticides and for guiding studies on biomarkers of early effects, which can be used later in programs to monitor the health of workers.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/metabo13050596/s1: Table S1—Gradient elution and flow rate used in chromatographic separation; Figure S1—OPLS-DA score graph for plasma samples from individuals exposed and non-exposed occupationally to pesticides; Figure S2—ROC curves of some of the discriminating plasma metabolites identified by liquid chromatography coupled with mass spectrometry, where AUC = area under the curve, with a 95% confidence interval; Figure S3—Orthogonal partial least squares discriminant analysis (OPLS-DA) for urine samples from individuals participating in the study; Figure S4—ROC curves of some discriminating urinary analytes identified by liquid chromatography coupled with mass spectrometry. Note: AUC: area under the ROC curve, with a 95% confidence interval.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Federal University of Minas Gerais—UFMG (CAAE: 39339720.0.0000.5149, opinion number: 5.473.586) for studies involving humans.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in article and supplementary material.

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