Luiza Flavia Veiga Francisco

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Orientador: Prof. Dr. Henrique César Santejo Silveira
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“Uma vida sem desafios não vale a pena ser vivida.”

Sócrates

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AUC	Acurácia
DDE	Diclorodifenildicloroetileno
DDT	Diclorodifeniltricloroetano
DGCR8	*DiGeorge syndrome critical region 8*
DLBCL	Linfoma difuso de grandes células B
DNA	Ácido desoxirribonucleico
HTLV1	Vírus linfotrópico de células T humanas tipo 1
LC-MS/MS	Cromatografia Líquida acoplada à Espectrometria de Massa em Tandem
LLC	Leucemia Linfocítica Crônica
LNH	Linfoma Não Hodgkin
MCN	Micronúcleo
miRISC	*miRNA-induced silencing complex*
miRNAs	microRNAs
MM	Mieloma Múltiplo
OR	Odds ratio
pre-miRNAs	*Precursor-miRNAs*
pri-miRNA	*primary-microRNA*
RNA	Ácido ribonucleico

LISTA DE SÍMBOLOS

<	Menor
≥	Maior ou igual

RESUMO

Francisco, LFV. Avaliação de biomarcadores em indivíduos expostos a agrotóxicos: uma abordagem exposômica. Tese (Doutorado). Barretos: Hospital de Câncer de Barretos; 2024.

O aumento da contaminação ambiental em grande parte está relacionado com as atividades antrópicas. Uma importante fonte desta contaminação é a agricultura. Os resíduos destes produtos contaminam o solo, ar e a água e, com isso, apresentam risco direto ou indireto a saúde humana e ambiental. A exposição aguda e crônica aos agrotóxicos está relacionada com diversos efeitos negativos à saúde humana, dentre eles, o aumento de risco de câncer, como os de Neoplasias de Células B Maduras. Diante disso, este estudo teve como objetivos: 1) realizar uma revisão sistemática para evidenciar a associação entre a exposição ocupacional a carcinógenos e o risco de Linfoma Não Hodgkin (LNH) e seus subtipos; 2) avaliar biomarcadores de exposição e efeitos associados a exposição ocupacional aos agrotóxicos em indivíduos saudáveis e pacientes com Neoplasias de Células B Maduras. Para isso, uma meta- análise foi conduzida para identificar as classes de trabalhadores que foram ocupacionalmente expostos a substâncias carcinogênicas potencialmente associados ao desenvolvimento de Linfoma não Hodgkin. Nossa revisão de literatura resultou em 2.719 artigos dos quais 51 foram incluídos na meta-análise, resultando em uma OR geral de 1,27 (IC 95% 1,03-1,55). Dentre esses trabalhos a principal classe trabalhadora associada com o aumento significativo do risco de LNH foram os trabalhadores expostos a agrotóxicos (14 estudos). Este estudo evidencia a associação positiva entre a exposição ocupacional a algumas substâncias, principalmente os agrotóxicos e o desenvolvimento de LNH. Para avaliar os biomarcadores associados a exposição ocupacional aos agrotóxicos, foram recrutados 46 indivíduos saudáveis (23 expostos e 23 não expostos ocupacionalmente a agrotóxicos) e 24 indivíduos com Neoplasias de Células B Maduras (13 expostos e 11 não expostos a agrotóxicos). Inicialmente, foi realizada a análise da quantificação de agrotóxicos no soro dos trabalhadores saudáveis expostos e não expostos ocupacionalmente utilizando o método GC-MS. Como resultado da análise, somente foi observada diferença significativa entre a quantificação do composto diclorodifenildicloroetileno (DDE) entre os trabalhadores expostos e não expostos, contudo, para os demais agrotóxicos, foi observada maior quantificação no grupo de trabalhadores

expostos ocupacionalmente quando comparado aos indivíduos não expostos. Em relação as análises de biomarcadores de efeito, inicialmente, os parâmetros de micronúcleo, brotamento, células binucleadas, em picnose e cariólise foram analisados para avaliar os efeitos genotóxicos, de falha na citocinese e morte celular entre os indivíduos saudáveis expostos e não expostos a agrotóxicos. De acordo com a análise realizada, os indivíduos do grupo exposto a agrotóxicos apresentaram significativamente um maior número de células com micronúcleo, binucleadas, picnóticas e em cariólise, quando comparado ao grupo não exposto ($p < 0,005$). Posteriormente realizamos a análise de perfil de expressão de microRNAs (miRNAs) pela tecnologia *NanoString*. Um total de 20 miRNAs com expressão diferencial significativa ($p < 0,005$) foram selecionados após análise da curva ROC (AUC ≥ 0,75) dentre os 30 miRNAs identificados com expressão diferencial entre as amostras de plasma do grupo exposto e não exposto. Quando comparado com o grupo não exposto, os 20 miRNAs foram regulados negativamente no grupo exposto. Um total de 1004 genes alvos validados foram preditos para os miRNAs. A análise de enriquecimento funcional demonstrou 213 processos biológicos, 131 componentes celulares, 170 termos de função molecular, além de 91 vias e 107 doenças relacionadas com os alvos dos miRNAs diferencialmente expressos. Em relação ao grupo de pacientes com Neoplasias de Células B Maduras, a análise de expressão dos miRNAs diferencialmente expressos entre os grupos exposto e não exposto para os pacientes com Leucemia Linfocítica Crônica (LLC) e Mieloma Múltiplo (MM), revelou 10 miRNAs com expressão diferencial significativa ($p < 0,01$) entre os grupos, no qual, 2 miRNAs apresentaram aumento da expressão e 8 miRNAs expressão diminuída no grupo de pacientes expostos. Todos os miRNAs diferencialmente expressos apresentaram uma área sob a curva (AUC) ≥ 0.80. A análise de predição de genes alvos resultou em 1.217 genes alvos para os miRNAs regulados negativamente e 159 genes alvos para os miRNAs regulados positivamente. Na análise de enriquecimento funcional dos genes alvos dos miRNAs regulados negativamente, foram observados 302 processos biológicos, 104 termos de componente celular, 97 termos para função molecular. Além disso, 77 vias enriquecidas e 76 doenças também foram associadas. Em relação aos alvos preditos dos miRNAs regulados positivamente, foram observados 28 processos biológicos, 12 termos de componente celular e 13 termos para função molecular, 1 via e 3 doenças associadas significativamente com os genes alvos. Os resultados obtidos demonstram que os indivíduos saudáveis do grupo exposto a agrotóxicos apresentam maior

genotoxicidade, falha na citocinese e citotoxicidade do que os indivíduos não expostos. Além disso, a análise de expressão dos miRNAs dos indivíduos saudáveis e dos pacientes com Neoplasias de Células B Maduras expostos a agrotóxicos apresentou modulação diferente quando comparada a expressão do grupo não exposto. Como conclusão, nosso estudo de meta-análise forneceu evidências da associação entre exposições ocupacionais e o risco de desenvolvimento de LNH por meio de uma avaliação detalhada de estudos epidemiológicos. Além disso, resultados obtidos demonstraram estar diretamente relacionados ao fator exposição e que os indivíduos expostos estão mais suscetíveis a riscos na saúde do que a população não exposta a estes compostos. Ainda, podemos salientar que a identificação de biomarcadores epigenéticos em indivíduos expostos aos agrotóxicos, como os miRNAs, podem ter um impacto considerável na prevenção do desenvolvimento de múltiplas doenças, o que pode contribuir no futuro com a medicina ambiental de precisão.

PALAVRAS-CHAVE: Agrotóxicos; Neoplasias de Células B Maduras; miRNAs; exposição ocupacional; Linfoma Não Hodgkin; micronúcleo.

ABSTRACT

Francisco, LFV. Evaluation of biomarkers in individuals exposed to pesticides: an exposomic approach. Thesis (Doctorate). Barretos: Barretos Cancer Hospital; 2024.

The increase in environmental contamination is largely related to human activities. An important source of this contamination is agriculture. The residues of these products contaminate the soil, air and water and, therefore, present direct or indirect risks to human and environmental health. Acute and chronic exposure to pesticides is related to several negative effects on human health, among them, the increased risk of cancer, such as Mature B-Cell Neoplasms. Therefore, this study had the following objectives: 1) to conduct a systematic review to demonstrate the association between occupational exposure to carcinogens and the risk of Non-Hodgkin Lymphoma (NHL) and its subtypes; 2) to evaluate biomarkers of exposure and effects associated with occupational exposure to pesticides in healthy individuals and patients with Mature B-Cell Neoplasms. For this, a meta-analysis was conducted to identify the classes of workers who were occupationally exposed to carcinogenic substances potentially associated with the development of Non-Hodgkin Lymphoma. Our literature review yielded 2,719 articles, of which 51 were included in the meta-analysis, resulting in an overall OR of 1.27 (95% CI 1.03-1.55). Among these studies, the main working class associated with a significantly increased risk of NHL were workers exposed to pesticides (14 studies). This study highlights the positive association between occupational exposure to some substances, mainly pesticides, and the development of NHL. To evaluate the biomarkers associated with occupational exposure to pesticides, 46 healthy individuals (23 occupationally exposed and 23 not exposed to pesticides) and 24 individuals with Mature B-Cell Neoplasms (13 exposed and 11 not exposed to pesticides) were recruited. Initially, the quantification of pesticides in the serum of healthy workers exposed and not exposed occupationally was performed using the GC-MS method. As a result of the analysis, a significant difference was only observed between the quantification of the compound dichlorodiphenyldichloroethylene (DDE) between exposed and non-exposed workers; however, for the other pesticides, greater quantification was observed in the group of occupationally exposed workers when compared to non-exposed individuals. Regarding the analysis of effect biomarkers, initially, the parameters of micronucleus, budding, binucleated cells, pyknosis and karyolysis were analyzed

to evaluate the genotoxic effects, cytokinesis failure and cell death among healthy individuals exposed and not exposed to pesticides. According to the analysis performed, individuals in the group exposed to pesticides presented a significantly higher number of cells with micronucleus, binucleated, pyknotic and karyolysis, when compared to the unexposed group ($p < 0.005$). Subsequently, we performed the microRNA (miRNA) expression profile analysis using NanoString technology. A total of 20 miRNAs with significant differential expression ($p < 0.005$) were selected after analysis of the ROC curve (AUC ≥ 0.75) among the 30 miRNAs identified with differential expression between the plasma samples of the exposed and unexposed groups. When compared with the unexposed group, 20 miRNAs were downregulated in the exposed group. A total of 1004 validated target genes were predicted for the miRNAs. Functional enrichment analysis demonstrated 213 biological processes, 131 cellular components, 170 molecular function terms, in addition to 91 pathways and 107 diseases related to the targets of the differentially expressed miRNAs. Regarding the group of patients with Mature B-Cell Neoplasms, the expression analysis of the differentially expressed miRNAs between the exposed and unexposed groups for patients with Chronic Lymphocytic Leukemia (CLL) and Multiple Myeloma (MM), revealed 10 miRNAs with significant differential expression ($p < 0.01$) between the groups, in which 2 miRNAs showed increased expression and 8 miRNAs decreased expression in the exposed group of patients. All differentially expressed miRNAs showed an area under the curve (AUC) ≥ 0.80. Target gene prediction analysis resulted in 1,217 target genes for downregulated miRNAs and 159 target genes for upregulated miRNAs. In the functional enrichment analysis of target genes of downregulated miRNAs, 302 biological processes, 104 cellular component terms, and 97 molecular function terms were observed. In addition, 77 enriched pathways and 76 diseases were also associated. Regarding the predicted targets of upregulated miRNAs, 28 biological processes, 12 cellular component terms, and 13 molecular function terms, 1 pathway, and 3 diseases were significantly associated with the target genes. The results obtained demonstrate that healthy individuals in the group exposed to pesticides present greater genotoxicity, cytokinesis failure, and cytotoxicity than unexposed individuals. Furthermore, the analysis of miRNA expression in healthy individuals and patients with Mature B-Cell Neoplasms exposed to pesticides showed different modulation when compared to the expression of the unexposed group. In conclusion, our meta-analysis study provided evidence of the association between

occupational exposures and the risk of developing NHL through a detailed evaluation of epidemiological studies. Furthermore, the results obtained demonstrated that they are directly related to the exposure factor and that exposed individuals are more susceptible to health risks than the population not exposed to these compounds. Furthermore, we can emphasize that the identification of epigenetic biomarkers in individuals exposed to pesticides, such as miRNAs, can have a considerable impact on the prevention of the development of multiple diseases, which may contribute in the future to precision environmental medicine.

KEYWORDS: Pesticides; Mature B-Cell Neoplasms; miRNAs; occupational exposure; Non-Hodgkin's Lymphoma; micronucleus.

INTRODUÇÃO

O Brasil é um dos países que mais consome pesticidas no mundo, fato este, que pode estar relacionado com a maior demanda de alimentos devido ao aumento populacional [1]. Os pesticidas são produtos químicos usados largamente em todo o mundo na produção de diferentes culturas com a finalidade de protegê-las contra a ação danosa de seres vivos considerados nocivos [2]. Porém, o uso indiscriminado destes produtos está relacionado com a contaminação ambiental, bem como, com o aumento do risco de exposição ocupacional, que pode causar toxicidade e doenças nos seres humanos.

A entrada destes produtos no corpo humano pode ser através do contato, ingestão e inalação [3]. Sabe-se que o contato de pessoas ocupacionalmente expostas durante a produção, manuseio e aplicação destes compostos, os torna o grupo mais susceptível ao risco da exposição. Diferentemente deste grupo, a população em geral tem como via principal de exposição a ingestão de alimentos e águas contaminadas por pesticidas [4]. A crescente exposição aguda e crônica a estes produtos pelas diferentes vias de exposição, tem evidenciado um elevado risco a saúde humana, visto que, a exposição aos pesticidas tem sido associada a eventos de intoxicação, alterações no sistema nervoso, digestivo, circulatório e respiratório [6-8]. Além disso, há associação de risco aumentado para alguns tipos de câncer devido a exposição crônica a estes compostos. Como relatado na literatura, a exposição aos pesticidas é considerada como fator de risco para: câncer de mama, próstata, útero, melanoma, colorretal, bexiga e linfoma não-Hodgkin [9-16].

Resultados do *Agricultury Health Study,* um estudo de coorte realizado nos Estados Unidos, têm evidenciado que indivíduos ocupacionalmente expostos a múltiplos agroquímicos (pesticidas, fertilizantes, solventes, dentre outros) constituem um grupo de maior risco para o desenvolvimento de LNH. Além disso, resultados desta coorte demonstraram que populações expostas ocupacionalmente aos pesticidas terbufos, lindano, diclorodifeniltricloroetano (DDT), clordano, diazinon, permetrina, glifosato, atrazina, paraquat e deltametrina, tiveram risco aumentado para LNH (Tabela 1).

Tabela 1- Associação de pesticidas com o aumento do risco de LNH em estudos do *Agricultural Health Study*.

Pesticidas	Linfoma não-Hodgkin e sub-tipos	Referência
Terbufos	Linfoma linfocítico crônico/Linfoma linfocítico de pequenas células	Alavanja et al., 2014 [8]
Terbufos	Linfoma não-Hodgkin	Bonner et al., 2010 [17]; Leon et al., 2019 [18]
Lindano	Linfoma Folicular	Alavanja et al., 2014 [8]
Lindano	Linfoma não-Hodgkin	Purdue et al., 2007 [18]
DDT	Linfoma linfocítico crônico/Linfoma linfocítico de pequenas células	Alavanja et al., 2014 [8]
DDT	Mieloma múltiplo	Louis et al., 2017 [19]
Clordano	Mieloma múltiplo	Louis et al., 2017 [19]
Diazinon	Linfoma Folicular	Alavanja et al., 2014 [8]
Permetrina	Mieloma múltiplo	Alavanja et al., 2014 [8]; Rusiecki et al., 2009 [20]
Glifosato	Linfoma difuso de grandes células B	Leon et al., 2019 [18]
Glifosato	Mieloma múltiplo	De Roos et al., 2005 [21]
Atrazina	Mieloma múltiplo; Linfoma não-Hodgkin	Rusiecki et al., 2004 [22]
Paraquat	Linfoma não-Hodgkin	Park et al., 2009 [23]
Deltametrina	Linfoma linfocítico crônico/Linfoma linfocítico de pequenas células	Leon et al., 2019 [18]

1.1 Linfoma não-Hodgkin

O linfoma não-Hodgkin (LNH) é um grupo heterogêneo com mais de 20 tipos diferentes de doenças causadas pela proliferação de células (linfócitos) malignas do sistema linfático que se espalham de maneira desordenada [24],[25]. Usualmente se originam nos linfonodos (nodal), mas, devido o sistema linfático ser encontrado em todo o corpo, o LNH pode ser iniciado em qualquer órgão do corpo (extranodal) e ocorrer em qualquer faixa etária. De acordo com a evolução clínica ele pode ser categorizado como linfoma de baixo grau (indolente) ou de alto grau (agressivo), e, portanto, o planejamento clínico se difere entre as categorias. O linfoma indolente mais comum é o linfoma folicular, enquanto o linfoma agressivo mais comum é o linfoma difuso de grandes células B [26].

De acordo com as estimativas do Globocan [27] para o ano de 2020, houveram 19,3 milhões de novos casos de câncer e quase 10 milhões de mortes por câncer em todo o mundo. O linfoma não Hodgkin foi responsável por 544.000 novos casos e 260.000 mortes em 2020 entre todos os países. Segundo as estimativas do Globocan o LNH, para ambos os sexos, é a 12° neoplasia mais incidente e a 13° com maior mortalidade entre todos os cânceres no mundo, enquanto que para o Brasil o LNH ocupa a 15° posição para mais incidente e para o índice de mortalidade (Figura 1).

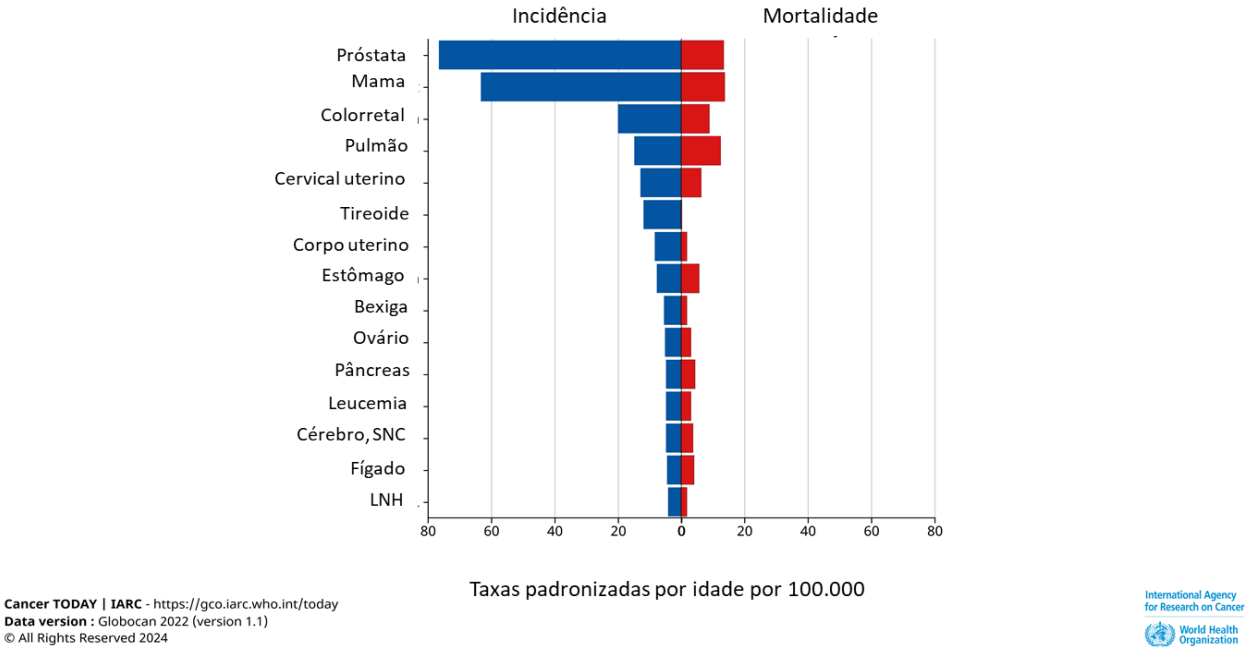

Figura 1- Número estimado de casos incidentes e mortes no Brasil para ambos os sexos e todas as idades. Fonte: Adaptado do Globocan, 2022.

No Brasil o Instituto Nacional de Câncer [25] estimou 6.420 novos casos de LNH em homens e 5.620 em mulheres para cada ano do triênio 2023-2025 (Figura 2). As causas do aumento da incidência do LNH permanecem em grande parte desconhecidas, porém, sabe-se que pessoas com o sistema imune comprometido, portadores do vírus Epstein-Barr e HTLV1 e da bactéria *Helicobacter pylori*, podem aumentar a chance de desenvolvimento de LNH. Além desses fatores, a exposição a altas doses de radiação, bem como a algumas substâncias químicas também constituem fatores de riscos para a doença [25].

Localização Primária	Casos	%
Próstata	71.730	30,0%
Cólon e reto	21.970	9,2%
Traqueia, brônquio e pulmão	18.020	7,5%
Estômago	13.340	5,6%
Cavidade oral	10.900	4,6%
Esôfago	8.200	3,4%
Bexiga	7.870	3,3%
Laringe	6.570	2,7%
Linfoma não Hodgkin	6.420	2,7%
Fígado	6.390	2,7%

Homens

Mulheres

Localização Primária	Casos	%
Mama feminina	73.610	30,1%
Cólon e reto	23.660	9,7%
Colo do útero	17.010	7,0%
Traqueia, brônquio e pulmão	14.540	6,0%
Glândula tireoide	14.160	5,8%
Estômago	8.140	3,3%
Corpo do útero	7.840	3,2%
Ovário	7.310	3,0%
Pâncreas	5.690	2,3%
Linfoma não Hodgkin	5.620	2,3%

*Números arredondados para múltiplos de 10.

Figura 2- Distribuição dos dez tipos de câncer mais incidentes para 2023 por sexo, exceto pele não melanoma.

Recentemente diversos estudos associaram o aumento do risco de subtipos específicos de LNH com poluentes ambientais. O risco para o subtipo Linfoma Folicular foi verificado aumentado para pessoas expostas ocupacionalmente ao tricloroetileno [28], benzeno, tolueno

e xileno [29]; Leucemia Linfocítica Crônica para exposição a tricloroetileno [28], benzeno, tolueno e xileno [29] e pesticidas [30]; e Mieloma Múltiplo com a exposição a pesticidas [31] e tricloroetano [32]. Numerosos estudos de revisão e meta-análise tem sido realizado com foco na associação entre agentes ambientais e LNH [33–36] , porém, nenhuma revisão com meta-análise foi publicada com estudos avaliando a associação entre um abrangente número de classe trabalhadora, substâncias carcinogênicas e o risco de LNH. A Revisão Sistemática é um tipo de estudo de alto nível de evidências disponíveis focadas em responder a uma pergunta específica. Esse tipo de estudo reúne em um único documento todas as pesquisas disponíveis, realiza a avaliação de estudos semelhantes a partir de trabalhos primários já publicados sobre assuntos específicos, através de métodos rigorosos, sistêmicos e explícitos [37,38]. Assim conduzir uma revisão sistemática com meta análise de estudos epidemiológicos observacionais para identificar classes de trabalhadores ocupacionalmente expostas a substâncias carcinogênicas associadas ao risco de LNH seria de extrema importância. Este tipo de estudo pode fornecer informações sobre a associação entre a exposição a pesticidas e o desenvolvimento de LNH. Pois, considerando o amplo uso de pesticidas no país, bem como, os efeitos negativos a saúde já relatada na literatura para alguns destes produtos, há grande preocupação com os impactos que os mesmos podem causar na saúde humana e ambiental. Apesar dos estudos anteriormente mencionados, ainda não se conhece o possível mecanismo que induz a carcinogênese causada pela exposição aos pesticidas.

1.2 Os pesticidas e as alterações celulares

Dentre os mecanismos dos pesticidas nas células, há a ocorrência de alteração metabólica, molecular e celular, indução de sinais inflamatórios e espécies reativas de oxigênio, os quais podem causar estresse oxidativo, ocasionando modificações de proteínas, disfunção mitocondrial, danos genéticos e doenças, como o câncer [39,40] (Figura 3).

Além disso, os pesticidas podem interferir na ação de hormônios endócrinos, uma vez que, podem mimetizar, interromper sua atividade, tempo de liberação, se ligar, ativar ou bloquear receptores de hormônios, interferindo em sua ação natural [39]. Em relação ao material genético, a interação com estes compostos muitas vezes ocasiona a instabilidade genômica, proporcionada pelas mutações no DNA, como quebras na cadeia, inserções, deleções, inversões e translocações, nos cromossomos, o que inclui alterações numéricas ou estruturais

e danos as proteínas. A instabilidade genômica é considerada como principal mecanismo envolvido na carcinogênese [39,41,42]. Entretanto, eventos epigenéticos, como a metilação do DNA, modificações nas histonas e alteração da expressão de miRNAs, são eventos celulares já estudados que alteram os perfis da expressão gênica e tem uma ligação intrínseca no processo de carcinogênese [6,39,40,43,44] (Figura 3).

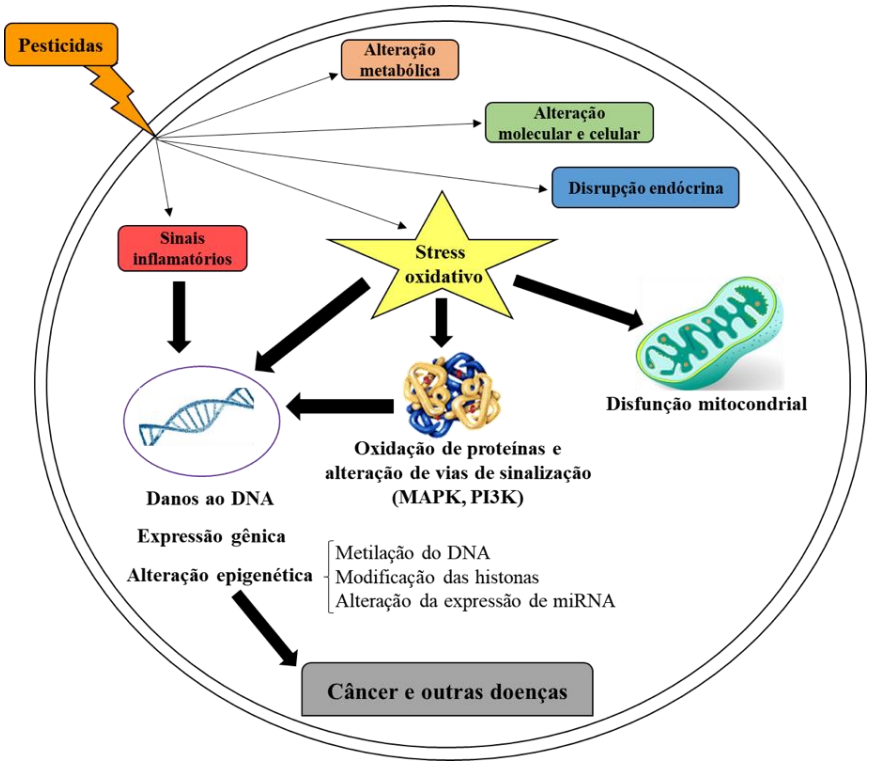

Figura 3- Efeitos da interação dos pesticidas com as células. Fonte: Adaptado de Sabarwal et al. (2018) e Silva; Jasiulionis (2014) [39,40].

Diante do exposto, uma abordagem exposômica com foco específico na exposição aos pesticidas vinculada a utilização de metodologias de avaliação genômica em larga escala, aliando essa exposição as mudanças bioquímicas e moleculares no corpo, poderá auxiliar na identificação de novos biomarcadores, o que pode levar a melhores estratégias que contribuam para prevenção de câncer. Além disso, tal abordagem pode contribuir para os estudos de nova geração de avaliação da exposição a químicos ambientais e suas misturas [45].

O *exposoma* é um conceito no qual refere-se à totalidade de exposições ambientais e/ou ocupacionais do indivíduo desde a concepção, o que compreende todas as exposições de origem interna e externa [35-37]. As exposições internas incluem as respostas biológicas

associadas a exposição a agentes ambientais e/ou ocupacionais, como, os processos endógenos (metabolismo, hormônios, inflamação, morfologia corporal, atividade física, microbiota intestinal, inflamação e envelhecimento). Por outro lado, as exposições externas podem ser gerais (ambiente urbano, fatores climáticos, sociais e de estresse) e específicas, que incluem diferentes fatores ambientais e estilo de vida (produtos químicos, agentes infecciosos, dieta, tabaco e álcool) [47,48].

Sendo assim, é importante que se realizem avaliações mais detalhadas dos riscos genotóxicos e epigenéticos causados pela exposição aos indivíduos ocupacionalmente aos pesticidas por meio do biomonitoramento, a fim de melhorar a qualidade de vida dessas pessoas e prevenir cânceres que podem ser relacionados ao ambiente de trabalho.

1.3 Instabilidade genômica na exposição ocupacional e/ou ambiental

A instabilidade genômica é definida como a capacidade aumentada do genoma em adquirir ou sofrer mutações. Sua ocorrência está relacionada com a disfuncionalidade de processos que realizam a manutenção e replicação do genoma [49]. Os mecanismos envolvidos nesse processo incluem defeitos herdados ou adquiridos no reparo do DNA, replicação do DNA, controle do ciclo celular ou segregação dos cromossomos [41]. Diante disso e do fato de muitas doenças e patologias exibirem instabilidade genômica, como, instabilidade de nucleotídeos (substituição de base, deleções e inserções de nucleotídeos), microssatélites (expansão ou contração de pequenas repetições de nucleotídeos) e de cromossomos (alteração no número e estrutura) [42,50], atualmente, a detecção dessa instabilidade pode ser obtida utilizando diversas tecnologias.

Um ensaio muito utilizado para avaliação da instabilidade genômica ocasionada pela exposição ambiental e/ou ocupacional é o ensaio de micronúcleo (MNC). O MNC é um biomarcador que corresponde a um pequeno núcleo arredondado disperso no citoplasma de células que sofreram a ação de agentes clastogênicos, promotores de quebras cromossômicas, e/ou aneugênicos, que induzem a perda ou a não disjunção de cromossomos inteiros na anáfase da divisão celular [51,52] (Figura 4).

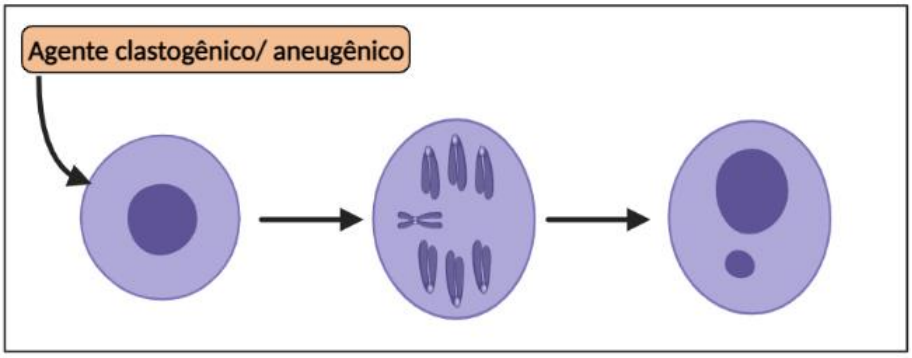

Figura 4- Formação do micronúcleo após exposição a agentes clastogênicos/ aneugênicos. Fonte: Próprio autor

Em diversos estudos que avaliaram a quantidade de MNC em pessoas expostas e não expostas ocupacionalmente a químicos, foi relatado aumento significativo no número de MNC em linfócitos ou células da mucosa bucal quando expostas a pesticidas [43-46], cigarro [56], petróleo e derivados [57], metais [58,59] e radiação [60,61]. Contudo, como dito anteriormente, além das alterações genéticas, os eventos epigenéticos constituem uma ferramenta importante no estudo dos mecanismos de carcinogênese promovido pela exposição ocupacional e ambiental.

1.4 Efeitos epigenéticos na exposição ocupacional e/ou ambiental

O evento epigenético refere-se à alteração na expressão gênica sem que ocorra modificações na sequência do DNA. Os mecanismos envolvidos incluem a metilação do DNA, modificação das histonas, remodelamento da cromatina e alteração da expressão de RNA não codificantes (miRNA) [62,63].

1.5 MicroRNAs

Os miRNAs correspondem a uma classe de RNAs não codificantes, de cadeia simples e com aproximadamente 22 nucleotídeos. São responsáveis por atuarem como reguladores da expressão gênica de modo pós-transcricional, inibindo o processo de tradução ou degradando o RNAm alvo [74-76] (Figura 5).

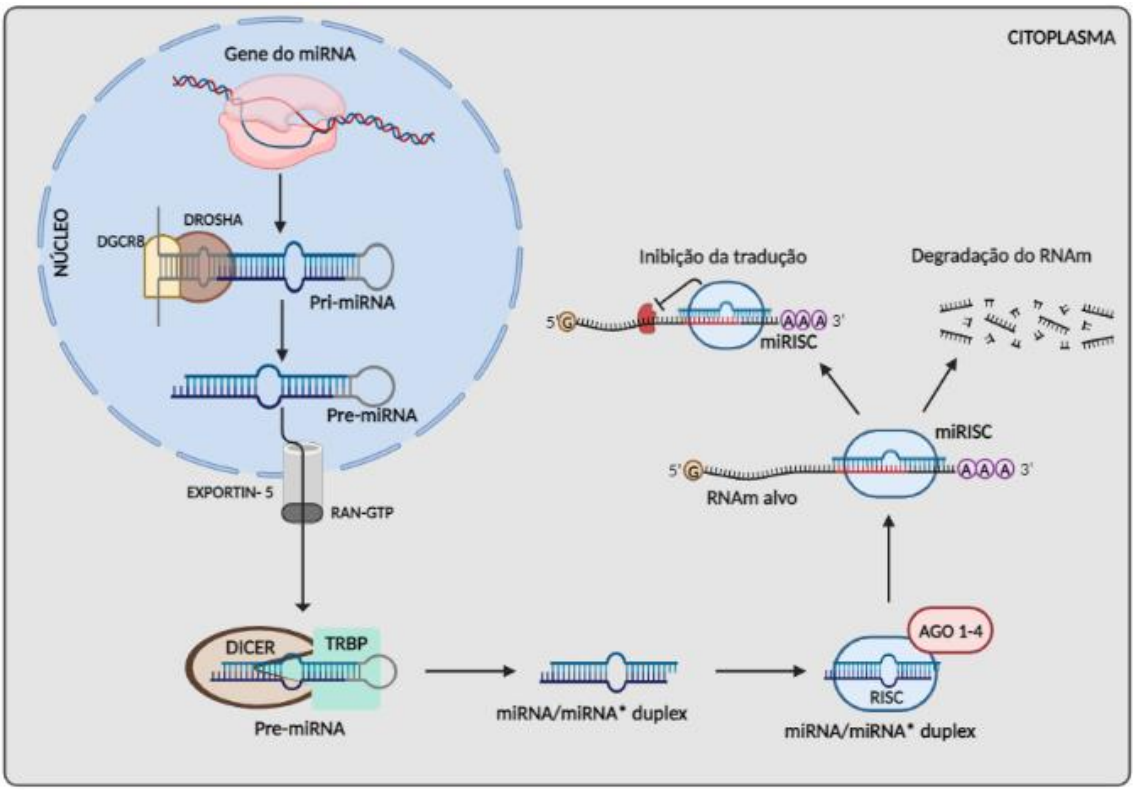

Figura 5- Biogênese dos microRNAs e mecanismos de regulação do mRNA-alvo. Os genes dos miRNAs são transcritos em miRNAs primários (pri-miRNA) que são processados no núcleo pelo complexo Drosha e DGCR8, dando origem aos miRNAs precursores (pre-miRNAs). Posteriormente os pre-miRNAs são exportados para o citoplasma da célula pela exportina-5. No citoplasma, a enzima Dicer e a proteína TRBP realizam a clivagem do pre-miRNA gerando um RNA de dupla fita (miRNA duplex). O miRNA maduro se associa ao complexo de silenciamento induzido por miRNA (miRISC) e são direcionados até ao mRNA alvo, podendo induzir o bloqueio da tradução ou ocasionar a degradação do mRNA, dependendo do grau de complementaridade. Fonte: Próprio autor

Devido sua estabilidade em temperatura ambiente e presença nos fluidos corporais, os miRNAs estão sendo considerados potenciais biomarcadores em estudos que envolvem biópsias líquidas incluindo o monitoramento de exposição ocupacional e doenças [77-79].

Dessa forma, estudos de regulação da expressão diferencial de miRNAs que visam a avaliação da influência da exposição ocupacional a poluentes e o envolvimento na patogênese de doenças, incluindo o câncer foram realizados recentemente e é um campo de exploração atual na investigação e mecanismos e descobrimento de novos biomarcadores envolvidos no câncer [70,71]. Análises para avaliação do perfil de expressão diferencial de miRNAs demonstraram alguns miRNAs com expressão alterada em pessoas expostas ocupacionalmente a vários compostos químicos.

No estudo realizado por Weldon et al. (2016) [66] avaliando potenciais miRNAS detectados na urina como biomarcadores da exposição a pesticidas, foi identificado seis

miRNAs associados ao trabalho agrícola. Associação de miRNAs com exposição ocupacional também foi verificada para solventes orgânicos (etilbenzeno, tolueno, xileno), no qual, miR-6819-5p e miR-6778-5p apresentaram regulação positiva em trabalhadores expostos em relação ao grupo não exposto [70]. Em um estudo com a avaliação de poluentes orgânicos persistentes, 93 miRNA demonstraram associação significativa com a exposição, dentre eles, miR-193a-3p, miR-152, miR-31-5p e miR-34a-5p, os quais são miRNAs supressores de tumor [72]. O aumento significativo da expressão de miR-222 e miR-21 foi observado após a exposição a material particulado [73].

Em trabalhadores expostos ao asbesto, 15 miRNAs diferencialmente expressos foram observados quando comparados com o grupo controle, sendo, hsa-miR-3960, hsa-miR-4497, hsa-miR-4508, hsa-miR-6089, hsa-miR-6125, hsa-miR-6775-5p e hsa-miR-6865-5p regulados negativamente [74]. Feng et al. (2017) [75] avaliando a exposição ao cloreto de vinila, sugeriram que miR-222-3p, miR-146a-5p, miR-151a-5p e miR-22-3p podem ser considerados biomarcadores da exposição. Neste estudo eles identificaram oito miRNAs regulados negativamente e sete positivamente no grupo altamente exposto, o miR-22-3p além de diferencialmente expresso no grupo exposto, também estava correlacionado com a frequência de micronúcleos. Outros estudos como, metais [65],[76], radiação [77], bifenilos policlorados [78], ozônio [79] e cigarro [80],[81] também foram realizados para investigação do perfil de miRNAs.

Diversos estudos têm demonstrado o papel dos miRNA como biomarcadores no LNH [92-96]. Os miR-21, miR-155 e miR-210 foram descritos como biomarcadores no linfoma difuso de grandes células B (DLBCL) [87], a superexpressão do miR-34a foi associada a um mal prognóstico de DLBCL [82]. De acordo Arzuaga-Mendez et al. (2021) [85], a superexpressão de miR-155-5p e miR-9-3p pode servir como biomarcadores para o diagnóstico de linfoma folicular. Por outro lado, diminuição dos níveis de expressão de miRNA-15a e miRNA-16-1 foi relatada em pacientes com LLC, o que pode servir como potencial biomarcador de diagnóstico [84].

Diante destes estudos, o uso de tecnologias para biomonitorar a exposição ocupacional pode contribuir para a prevenção do câncer, bem como, ajudar na compreensão do papel molecular dos compostos químicos na doença e na revelação de novos biomarcadores. Além disso a avaliação de populações expostas e a utilização de tecnologias em larga escala para elucidar assinaturas epigenéticas contribuem para a nova área conhecida como *exposoma*.

2 JUSTIFICATIVA

O grupo mais susceptível aos efeitos toxicológicos ou patológicos destes produtos são os trabalhadores rurais expostos aos pesticidas, pois, apresentam contato direto com vários princípios ativos, sendo que, alguns são classificados pela Organização Mundial de Saúde como potenciais carcinogênicos. De acordo com os estudos epidemiológicos que associam o aumento do número de casos de câncer com a exposição ocupacional a pesticidas, faz-se de extrema importância a realização da avaliação da população exposta diariamente aos pesticidas, por meio de testes de instabilidade genômica e dos efeitos epigenéticos, afim de correlacioná-los com a exposição. Além disso, este projeto se aplica ao contexto do *exposoma*, uma abordagem que avalia por biomonitoramento um determinado perfil de exposição e visa a identificação de biomarcadores biológicos para fornecer novas informações sobre a exposição e os efeitos fisiopatológicos precoces subsequentes, por metodologias em larga escala.

Por outro lado, considerando que as Neoplasias de Células B Maduras constituem um dos tipos de cânceres mais comuns associados a exposição ocupacional a pesticidas, a realização de uma revisão sistemática com meta-análise que investigue a associação da exposição ocupacional a substâncias químicas, dentre eles, os pesticidas com o desenvolvimento de LNH, se torna de extrema importância para o entendimento dessa relação e direcionamento de estudos moleculares futuros. Além disso, a realização de estudos moleculares envolvendo pacientes com este tipo de câncer que foram expostos anteriormente a pesticidas, auxiliará na elucidação do papel da exposição ocupacional na doença. Assim, este estudo poderá identificar novos biomarcadores na resposta precoce da exposição ocupacional aos pesticidas.

3 OBJETIVOS

3.1 Objetivo Geral

- Avaliar biomarcadores associados a exposição ocupacional aos pesticidas em indivíduos saudáveis e com Neoplasias de Células B Maduras expostos a pesticidas.

3.2 Objetivos específicos

- Realizar uma revisão sistemática com meta-análise para verificar se há associação entre a exposição ocupacional e o risco de LNH.
- Avaliar e detectar os principais pesticidas (e metabólitos) no soro de indivíduos expostos e não expostos aos pesticidas, utilizando o método *GC-MS.*
- Avaliar a instabilidade genômica pelo ensaio de micronúcleo em amostras de mucosa oral de indivíduos saudáveis expostos e não expostos aos pesticidas.
- Determinar o perfil de expressão de microRNAs no plasma de indivíduos saudáveis e com câncer expostos e não expostos a pesticidas por meio da tecnologia *NanoString*.
- Predizer os genes-alvo e identificar vias moleculares associadas com os miRNAs apontados como diferencialmente expressos.
- Correlacionar os dados da expressão diferencial dos microRNAs e frequência de micronúcleos com os dados epidemiológicos dos indivíduos saudáveis.
- Correlacionar o padrão de expressão dos miRNAs apontados como potenciais biomarcadores com os dados clínicos e epidemiológicos dos indivíduos com LLC e MM.

4 RESULTADOS

4.1 Artigo 1- Occupational exposures and risk of Non-Hodgkin Lymphoma: a meta-analysis

cancers

MDPI

Review

Occupational Exposures and Risks of Non-Hodgkin Lymphoma: A Meta-Analysis

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Simple Summary: It is unclear what causes the increased incidence of non-Hodgkin lymphoma (NHL); however, chemical substance exposure is known to be one of the risk factors for the disease. The aim of our systematic review was to verify the association between occupational exposure to carcinogens and NHL risk. In our literature review, 51 articles were included in the meta-analysis resulting in an overall OR of 1.27 (95% CI 1.04–1.55). Among these studies, 20 reported a significant association with the increased risk of NHL. We demonstrate that the risk of NHL increases for individuals occupationally exposed to pesticides, benzene, and trichloroethylene. Our findings may provide information for public health and practical decision-making about certain work activities and the use of chemical compounds.

Abstract: Non-Hodgkin lymphoma (NHL) is a heterogeneous group with different types of diseases. It remains unclear as to what has led to an increase in incidences of NHL, however, chemical substance exposure is known to be one of the risk factors for the disease. Therefore, we performed a systematic review and meta-analysis including case-control, cohort, and cross-sectional observational epidemiological studies to verify the association between occupational exposure to carcinogens and NHL risk. Articles between the years 2000 and 2020 were collected. Two different reviewers performed a blind selection of the studies using the Rayyan QCRI web app. Post-completion, the selected articles were extracted and analyzed via the RedCap platform. Our review resulted in 2719 articles, of which 51 were included in the meta-analysis, resulting in an overall OR of 1.27 (95% CI 1.04–1.55). Furthermore, it was observed that the main occupation associated with the increased risk of NHL was that in which workers are exposed to pesticides. We therefore conclude that the evidence synthesis of the epidemiological literature supports an increased risk for NHL, regardless of subtype, considering occupational exposure to certain chemical compounds, mainly pesticides, benzene, and trichlorethylene, and certain classes of work, primarily in the field of agriculture.

Keywords: non-Hodgkin lymphoma; occupational exposure; pesticides; carcinogens

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1. Introduction

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of diseases that stem from the proliferation of malignant lymphocytes and their precursors. These cells accumulate in the lymph nodes, but can also extend to other organs [1,2]. There are different subtypes of NHL, often with variations in their presentations, prognoses, and clinical treatments [3]. NHL ranks as the eighth and tenth most frequent cancer among men and

women worldwide, respectively, with as many as 544,000 new cases and 260,000 deaths estimated for 2020 [4]. According to the data from Sung et al. [4] the highest incidence rates for NHL were found in Australia, New Zealand, North America, and Europe.

Etiologies of most NHL's remain largely unknown, however, risk factors are likely to include immune deficiencies, Epstein-Barr virus, HTLV1, and Helicobacter pylori bacteria, radiation, chemicals, such as benzene, persistent organic pollutants, and pesticides [5,6]. Several environmental exposures have been suggested and investigated as factors that can potentially lead to the increased risk of NHL and play a crucial role in the increase of incidence of cases.

Many recent studies have examined the association between environmental or occupational exposure to chemical compounds and the risk of developing NHL and specific subtypes [7–11]. In addition, many occupations have been investigated to establish the relationship between exposure and NHL risk [12–18]. Nonetheless, further studies are needed that evaluate the association between the increased risk of NHL and occupations in which chemical exposure is prevalent.

There have been several review and meta-analysis studies focusing on the association between environmental pollutants and NHL [19–22], however, as of yet, no such review with a meta-analysis has been published featuring studies evaluating the association between a comprehensive number of work classes, chemical agents, and the risk of NHL. In short, the evidence regarding the link between occupations and chemical compounds and the development of NHL has not been completely understood to date. As such, we have conducted a systematic review, through meta-analysis, of observational epidemiological case-control, cohort, and cross-sectional studies to identify occupational classes of workers exposed to chemical agents associated with risks of NHL.

2. Materials and Methods

The systematic review and meta-analysis were performed according to the guidelines specified by PRISMA [23,24]. The review protocol was registered in the PROSPERO database (ID: CRD42020160291).

2.1. Search Strategy

Meta analysis was conducted to identify which types of workers were occupationally exposed to chemical agents that were potentially linked to the development of non-Hodgkin lymphomas. A systematic literature search of articles in the Medline, Cochrane Library, Virtual Health Library-BVS Regional Gateway, Scopus, PubMed, and Embase databases, from 2000 to 2020, was conducted. The search included the keywords "environmental carcinogens", "iarc classification", "occupation cancer", "non-Hodgkin's lymphoma", "carcinogenic agents", "environmental factors", "occupational cancer", "occupational exposure", "workers". Details of this search strategy are reported in Supplementary Material.

2.2. Study Selection and Data Extraction

Studies that fulfilled the specific inclusion criteria were included in the meta-analysis: (a) case-control, cross-sectional, prospective cohort, and retrospective type observational epidemiological studies; (b) original studies based on workers exposed to carcinogens; studies whose outcome was NHL; (c) articles written in English only; (d) studies in which the association between occupational exposure and the risk of NHL were expressed as relative risk (RR), standardized mortality ratios (SMR), hazard risk (HR), standardized incidence ratios (SIR), proportional mortality ratio (PMR), odds ratio (OR) with 95% confidence intervals (CIs), either reported or could be obtained from the data reported in the article. Studies such as meta-analysis, reviews, meetings, abstracts, letters, and comments were not included in this review.

Study selection was performed independently by two reviewers using the blind system using the Rayyan QCRI online web app software [25] to categorize, include, and exclude eligible articles during the preliminary screening process based on the titles and abstracts.

After the initial screening step, the entire text of the articles, which may contain relevant information, was reviewed by the two reviewers. The following information was extracted from the eligible articles: authorship, year of publication, country of publication and study, study design, population, gender of participants, diagnosis, occupational activity, chemical agent, sample size, number of exposed and non-exposed individuals (cohort and cross-sectional studies), number of case and control (case-control study), number of exposed individuals who developed NHL, and outcome measures. The RedCap web platform was used for data extraction by both reviewers and tabulated electronically. During each step, the results were compared, and any discrepancies were clarified with the participation of a third reviewer.

2.3. Quality Assessment

Study quality assessment was carried out using the Study Quality Assessment Tool, developed by methodologists at the National Heart, Lung, and Blood Institute (NHLBI), in conjunction with professionals at the Research Triangle Institute International. The tool was based on other assessment tools created by the Evidence Based Practice Centers of the Agency for Healthcare Research and Quality (AHRQ), Cochrane, the Scottish Intercolle- giate Guidelines Network and, others working in the context of evidence-based medicine. Designed to assist reviewers, the tool was considered, for this research, fundamental to the critical evaluation of the internal validity of a study. This includes items to assess potential flaws in study methods, including risks of bias.

During the quality review of the studies, each item of the tool may be answered with a "yes", "no", or "cannot be determined/not reported/not applicable". For each item in which "no" was selected, a potential risk of bias was considered. This assessment tool is not designed to provide a list of factors that make up a numerical score or to delimit a score for reviewers. Thus, it is used, by following with the literature, for intervention studies, a rating of bad ≤ 6, regular > 6 and 5, and good ≥ 10 is used. For case-control studies that contained 12 questions instead of 14, the rating was adjusted: bad ≤ 5, regular > 5, and <9, and good ≥ 9 [26].

2.4. Data Analysis of the Systematic Review

Results from the cohort, cross-sectional, and case-control studies that associated exposure to chemical compounds or work class with increased risk of NHL were included in the meta-analysis. The meta-analysis was performed for each comparison using the random effects model, and the OR with the 95% Confidence Interval (CI) was calculated using the Mantel Haenszel method. The combined OR ($\hat{\psi}_{MH}$) was estimated by adding together the individual ORs ($\hat{\psi}_k$) from each study according to the expression of [27]:

$$\hat{\psi}_{MH} = \frac{\sum_{k=1}^{K} w_k \hat{\psi}_k}{\sum_{k=1}^{K} w_k} \quad (1)$$

where:

$w_k = (b_k\, c_k)/n_k$
K = total number of studies
$k = 1, 2, \ldots, K$
$\psi_k = (a_k\, d_k)/(b_k\, c_k)$
n_k = study sample size k

a_k = number of events in the group exposed in the study k
b_k = number of non-events in the group exposed in the study k
c_k = number of events in the unexposed group in the study k
d_k = number of non-events in the non-exposed group in the study k
n_k = sample size (all studies)

Variables included in the meta-analysis were sample size, number of exposed individuals, number of non-exposed individuals, number of exposed individuals with an event, and

non-exposed individuals with an event. Heterogeneity between each study was estimated using the Q-statistic of the Q-Cochran test and quantified using the inconsistency index (I^2) [28]. Funnel plot was used to assess potential publication bias [29]. Analyses were performed using R version 4.1.2 (1 November 2021) software, using the functions metabin, forest.meta, and funnel from the package meta.

3. Results and Discussion

3.1. Characterization of the Studies

The search of the database resulted in 2719 articles. Duplicate and irrelevant articles were excluded as described in the inclusion criteria. A total of 537 duplicate articles were excluded while the remaining 2182 were selected for evaluation based on titles and abstracts. Evaluation of the titles and abstracts revealed that 1854 articles did not analyze the association between occupations and carcinogens with the increased risk of NHL. As a result, an additional 1854 articles were excluded. The remaining 328 articles were subjected to a full-text review; of these, 27 articles did not meet the inclusion criteria. During data extraction, 129 articles were excluded by methodological evaluation, as a result, only 172 remained eligible for the study. Ultimately, 51 articles were included in the meta-analysis, because 121 articles did not present the necessary information for the calculation of the effect size (Odd Ratio) (Figure 1).

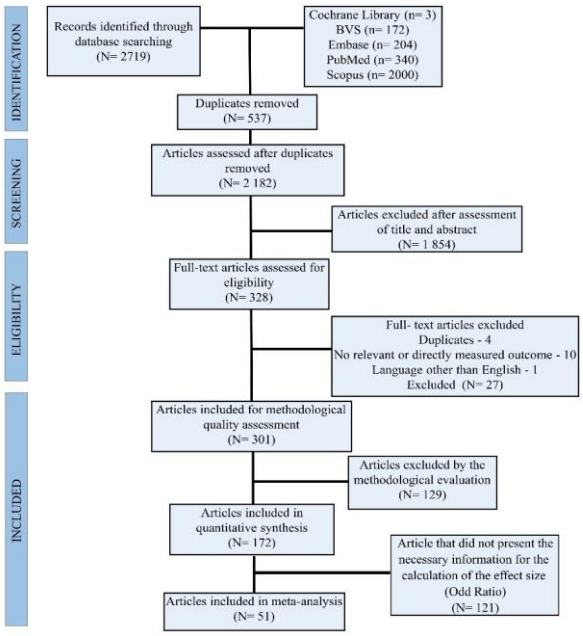

Figure 1. Flowchart of search and selection of articles included in the review and meta-analysis according to PRISMA.

The systematic review comprised 76 case-control articles and 96 cohort/transversal articles. In regards to geographic coverage, of the 172 articles included in this review, 74 studies were conducted in the United States, 17 in Canada, 14 in France, 13 in Italy and Sweden, 10 in Germany and Spain, 9 in Denmark and Norway, 7 in China and the United Kingdom, 6 in Australia, New Zealand, and the Czech Republic, 5 in Finland and Ireland, 4 in Japan, 3 in Brazil, England, and Iceland, 2 in Korea, Greece, the Netherlands, and Iran, 1 in South Africa, Singapore, Poland, Taiwan, and Ukraine (Figure 2).

Several studies conducted at these locations are cohort studies or are part of consortia. The Agricultural Health Study (AHS) was the main cohort study evaluated in the studies [30–42]. The AHS is a prospective study of licensed pesticide applicators and their spouses in Iowa and North Carolina (USA), designed to understand how agricultural, lifestyle, and genetic factors affect the health of agricultural populations. The International Lymphoma Epidemiology Consortium (InterLymph) was found to have the largest number of studies among the consortia analyzed for this review [8,13,14,18,43–45]. The InterLymph Consortium conducts research through case-control studies in non-Hodgkin lymphoma addressing genetics, immunity and infection, lifestyle and environment, pathology, and survival. These types of studies have contributed greatly over the years to the understand-

ing of the association between environmental/occupational exposure and the development of diseases such as non-Hodgkin lymphomas.

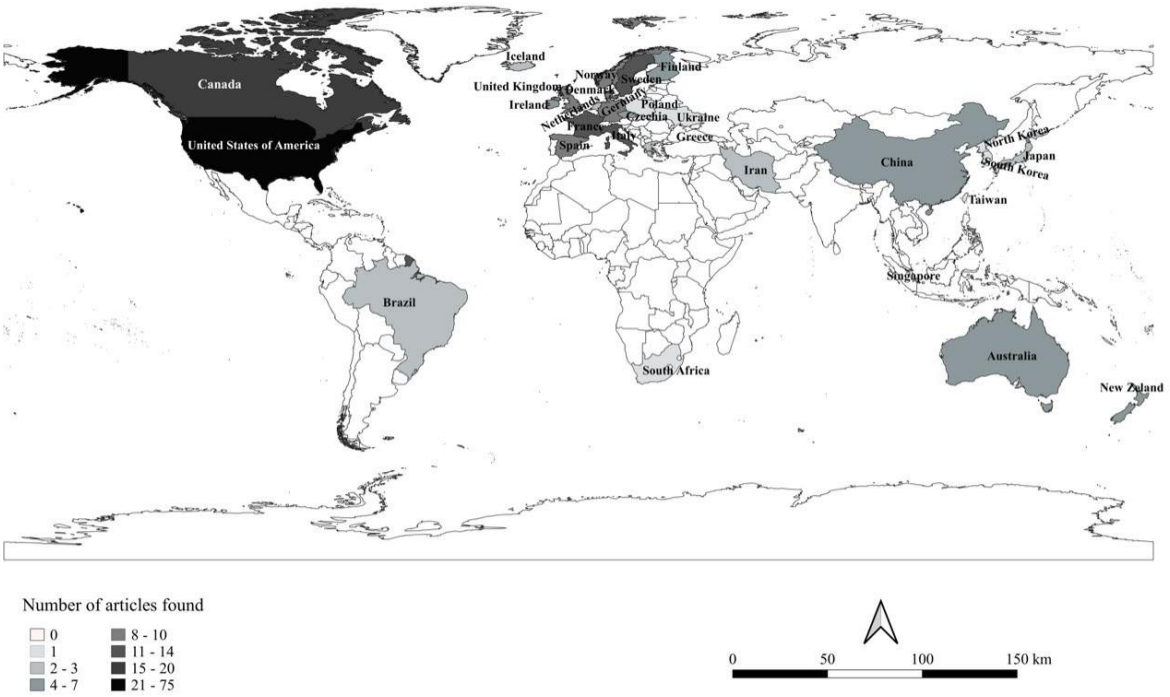

Figure 2. Location of studies included in the review that investigated the association of occupational exposure and NHL.

The association between a large number of work classes and chemical compounds associated with the development of NHL and its subtypes has not been evaluated in any review. Reviews typically evaluate the association between the increased risk of overall NHL or some specific subtype with only one group of chemical agents or work class [21,22,46,47]. In our review, the following occupations: painter, driver, construction worker, hairdresser, chemical industry employee, solvent-exposed employees, agricultural mechanics and laborers, husbandry workers, and fishing laborers were the most investigated work classes used in the studies included in the review. In terms of the chemical compounds investigated in the studies, the most frequently evaluated were pesticides, radiation, solvents, hydrocarbons, metals, organic compounds, asbestos, paints, petroleum products, and organochlorines.

3.2. Characteristics of the Studies Included in the Meta-Analysis

A total of 51 observational studies that met our inclusion criteria were identified and included in the meta-analysis. Accordingly, 28 case-control studies and 23 cohort studies were included. Table 1 shows an evaluated summary of the data extracted from each study, including author and year of publication, site, study design, exposure categories (work class and carcinogen), NHL subtype, and OR. Given the types of exposures present in the articles, the primary class of work evaluated in the articles, as part of the meta-analysis, was agricultural occupation and related activities. The main chemical agents analyzed were pesticides and solvents. When analyzing the NHL subtypes evaluated in the articles, of the 51 articles included in the meta-analysis, 22 evaluated the risk of Chronic Lymphocytic Leukemia/Small Cell Lymphocytic Lymphoma (CLL/SLL), 20 of Multiple Myeloma (MM), 17 of Follicular Lymphoma (FL) and Diffuse Large B Cell Lymphoma (DLBCL), and 6 of B cell in general. Although these were the most commonly investigated subtypes in the articles included in the meta-analysis, other subtypes of NHL were also evaluated as noted in Table 1.

Table 1. Characteristics of the studies included in the meta-analysis.

Authors	Country	Study Design	Work Class	Carcinogen	NHL Subtype	Odds Ration IC 95%
Benavente et al. (2020) [7]	Spain	Case-control	-	Pesticides	NHL; CLL	1.61 (1.22–2.12)
Fisher et al. (2020) [42]	USA	Cohort	- Pesticide Applicators	-	NHL; BC-NHL; CLL; SLL; DLBCL; FL; MM	4.05 (3.11–5.27)
Linet et al. (2020) [48]	China	Cohort		Benzene	NHL	2.27 (1.00–5.13)
Satta et al. (2020) [49]	Czech Republic, France, Germany, Ireland, Italy and Spain	Case-control	-	Internal and External Ionizing Radiation	BC-NHL; CLL; DLBCL; FL; MM	0.87 (0.73–1.05)
Loomis et al. (2019) [50]	Denmark, Finland, Italy, Norway, Sweden, and the United Kingdom	Cohort	- Workers in the reinforced plastic industry - Laminators	Styrene	NHL; MM	0.61 (0.27–1.38)
Jordan et al. (2018) [51]	USA	Cohort	- Search/Rescue Workers	-	NHL	0.44 (0.22–0.86)
McBride et al. (2018) [52]	New Zealand	Cohort	-	2,3,7,8-tetraclorodibenzo-*p*-dioxin (TCDD)	NHL; MM	0.51 (0.14–1.91)
Tsai et al. (2018) [53]	Taiwan	Cohort	- Farmers	-	NHL	1.16 (1.0–1.33)
Boccolini et al. (2017) [54]	Brazil	Case-control	- Farm Workers	-	NHL	0.88 (0.75–1.02)
Ferri et al. (2017) [55]	Italy	Case-control	- Homemaker - Blue-collar worker - Teachers - Craftsman/Merchant - Farmer - Clerk - Military - Technician - Food handlers - Agricultural occupation	Pesticides and radon	NHL; DLBCL; FL; CLL; Single B-cell Lymphoma; MM	2.31 (0.73–7.32)
Lemarchand et al. (2017) [56]	France	Cohort	- Farm Workers		NHL; CLL/SLL; FL; DLBCL; MCL; MZL; Waldenström Lymphoplasmacytic Lymphoma; NK/T-CL; MF; NHL-NOS	1.23 (0.99–1.54)
González et al. (2016) [57]	USA	Cohort	- Radiologist		NHL; CLL; MM	0.95 (0.77–1.17)
Bassig et al. (2015) [58]	China	Cohort	- Factory Employees Exposed to Benzene	Benzene	NHL	1.78 (1.13–2.81)
Cocco et al. (2013) [8]	USA, Czech Republic, France, Germany, Italy, Ireland, Spain and Canada	Case-control	-	Trichloroethylene	NHL; DLBCL; FL; CLL	1.01 (0.86–1.17)
Kachuri et al. (2013) [59]	Canada	Case-control	- Farm workers	Pesticides and formaldehyde	MM	1.09 (0.84–1.41)
Karunanayake et al. (2013) [60]	Canada	Case-control	- Workers exposed to Pesticides	-	NHL	1.79 (1.0–3.18)
Li et al. (2012) [61]	USA	Cohort	- Search/Rescue Workers		NHL; MM	1.26 (0.81–1.98)

Table 1. *Cont.*

Authors	Country	Study Design	Work Class	Carcinogen	NHL Subtype	Odds Ration IC 95%
Zakerinia et al. (2012) [11]	Iran	Case-control	- Workers exposed to Pesticides	-	NHL; MM	5.48 (3.47–8.65)
Lipworth et al. (2011) [62]	USA	Cohort	- Employees of aircraft manufacturing - Aircraft painter - Process or electroplating operator - Plastic parts manufacturer - Welder - Metal bonding worker - Fabrication and structural development mechanic - Final Assembler	Chromate and solvents	NHL; MM; CLL	1.20 (1.02–1.40)
Purdue et al. (2011) [63]	USA	Case-control	-	Trichloroethylene	NHL; DLBCL; FL; SLL; CLL	1.20 (1.01–1.42)
Bonner et al. (2010) [32]	USA	Cohort	-	Terbufos	NHL	1.39 (0.97–1.97)
Cocco et al. (2010) [9]	Czech Republic, France, Germany, Ireland, Italy, and Spain	Case-control	-	Solvents	DLBCL; FL; CLL; MM; BC- NHL	1.09 (0.97–1.23)
Kiran et al. (2010) [64]	Czech Republic, France, Germany, Italy, Ireland, and Spain	Case-control	-	Ethylene	DLBCL; CLL	1.21 (0.72–2.03)
Seidler et al. (2010) [65]	Germany, and Italy	Case-control	-	Asbestos	BC-NHL; DLBCL; FL; CLL; MM	1.11 (0.83–1.47)
Wong et al. (2010) [66]	China e USA	Case-control	- Farmer -Livestock or Animal Husbandry	-	NHL; CLL; FL; DLBCL; T/NK Cell Neoplasms	1.37 (1.11–1.69)
Lynch et al. (2009) [37]	USA	Cohort	-	Butylate	NHL	1.81 (1.0–3.30)
McBride et al. (2009) [67]	New Zealand	Cohort	-	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	NHL; MM	2.05 (0.24–17.64)
Park et al. (2009) [39]	USA	Cohort	-	Paraquat	NHL	1.15 (0.80–1.66)
Rusiecki et al. (2009) [41]	USA	Cohort	- Farmers	-	NHL; MM	0.08 (0.06–0.12)
Wang et al. (2009) [68]	USA	Case-control	-	Solvent	NHL; DLBCL; FL; CLL	1.61 (1.29–2.01)
Kirkeleit et al. (2008) [69]	Norway	Cohort	- Oil industry workers	-	NHL; CLL; MM	0.67 (0.48–0.93)
Tranah et al. (2008) [70]	USA	Case-control	- Animal Husbandry - Farm Laborer	-	NHL; DLBCL or Immunoblastic Large Cell Lymphoma; FL; SLL	0.93 (0.81–1.07)
Bonner et al. (2007) [33]	USA	Cohort	-	Malathion	NHL	10.10 (5.18–19.68)
Mahajan et al. (2007) [38]	USA	Cohort	-	Carbaryl	NHL	14.67 (8.19–26.25)
Moore et al. (2007) [71]	Czech Republic, France, Germany, Ireland, Italy, and Spain	Case-control	- Meat handlers	-	NHL; MM; CLL; SLL; DLBCL; FL	1.23 (1.00–1.51)

Table 1. *Cont.*

Authors	Country	Study Design	Work Class	Carcinogen	NHL Subtype	Odds Ration IC 95%
Purdue et al. (2007) [40]	USA	Cohort	-	Pesticides	NHL	2.55 (1.39–4.70)
Xu et al. (2007) [72]	Japan, Korea and China	Case-control	- Farmers - Chemical Plant Workers - Self-Employed	Pesticides	Nasal T/NK-cell Lymphoma	0.81 (0.49–1.34)
Miligi et al. (2006) [73]	Italy	Case-control	-	Solvents and hydrocarbons	NHL; SLL; FL; DLBCL	1.11 (0.92–1.33)
Balen et al. (2006) [12]	Spain	Case-control	- Farmer - Animal Husbandry	-	NHL; B-Cell NHL; TC-NHL; MM	1.34 (1.08–1.66)
Fritschi et al. (2005) [74]	Australia	Case-control	-	Pesticides	NHL; BC- NHL; DLBCL; FL	1.00 (0.71–1.41)
Kato et al. (2005) [75]	USA	Case-control	-	Solvents	NHL	1.18 (0.85–1.63)
Chiu et al. (2004) [76]	USA	Case-control	-	Pesticides	NHL; DCL; SLL; FL	1.11 (0.94–1.32)
Kato et al. (2004) [77]	USA	Case-control	- Farm Workers - Pesticide Applicators	Pesticides and naphthalene	NHL	1.40 (1.01–1.94)
De Roos et al. (2003) [78]	USA	Case-control	-	Pesticides	NHL	1.39 (1.16–1.67)
Fritschi et al. (2002) [79]	Canada	Case-control	- Workers in contact with animals - Farm Workers - Fishermen	-	NHL; MM	0.92 (0.79–1.08)
Lee et al. (2002) [80]	USA	Cohort	- Farmers - Cattle Ranchers	-	NHL; MM; CLL	0.99 (0.65–1.51)
McDuffie et al. (2001) [81]	Canada	Case-control	-	Pesticides	NHL	2.56 (2.09–3.14)
VanWijngaarden and Savitz (2001) [82]	USA	Case-control	- Electric Utility Workers - Electric Utility Workers Exposed to Solvents	-	NHL; Low and high-grade NHL	1.24 (0.85–1.81)
Zheng et al. (2001) [83]	USA	Case-control	-	Pesticides	NHL; FL; Diffuse NHL; SLL	0.42 (0.34–0.51)
McGeoghegan and Binks (2000) [84]	England	Cohort	- Uranium Production and Manufacturing Workers	Radiation	NHL; MM	1.14 (0.53–2.43)
Thörn et al. (2000) [85]	Sweden	Cohort	- Lumberjack	-	NHL	0.95 (0.13–6.77)

NHL: Non-Hodgkin's Lymphoma; CLL: Chronic Lymphocytic Leukemia; BC-NHL: B-Cell Non-Hodgkin's Lymphoma; SLL: Small Cell Lymphocytic Lymphoma; DLBCL: Diffuse Large B Cell Lymphoma; FL: Follicular Lymphoma; MM: Multiple Myeloma; MCL: Mantle Cell Lymphoma; MZL: Marginal Zone Lymphoma; NK/T-CL: NK/T-cell Lymphoma; MF: Mycosis Fungoides; TC-NHL: T-Cell Non-Hodgkin's Lymphoma; DCL: Diffuse Cell Lymphoma; USA: United States of America.

The meta-analysis of the 51 studies produced an overall OR of 1.27 (95% CI 1.03–1.55). This suggests that some work classes and occupational exposure to certain compounds are associated with a 27% increase in the risk of NHL. The highest OR entered was for the study by Mahajan et al. [38] (OR = 14.67), in which they assessed exposure to pesticides. The lowest was for the study by Rusiecki et al. [41] (OR = 0.08), in which they assessed individuals in agricultural occupations. No individual study received more than 3% of the total weight assigned in the random effects model (Figure 3). The random effects model yielded a heterogeneity value of 93% with $p < 0.01$, indicating significant heterogeneity across studies (Figures 3 and S1).

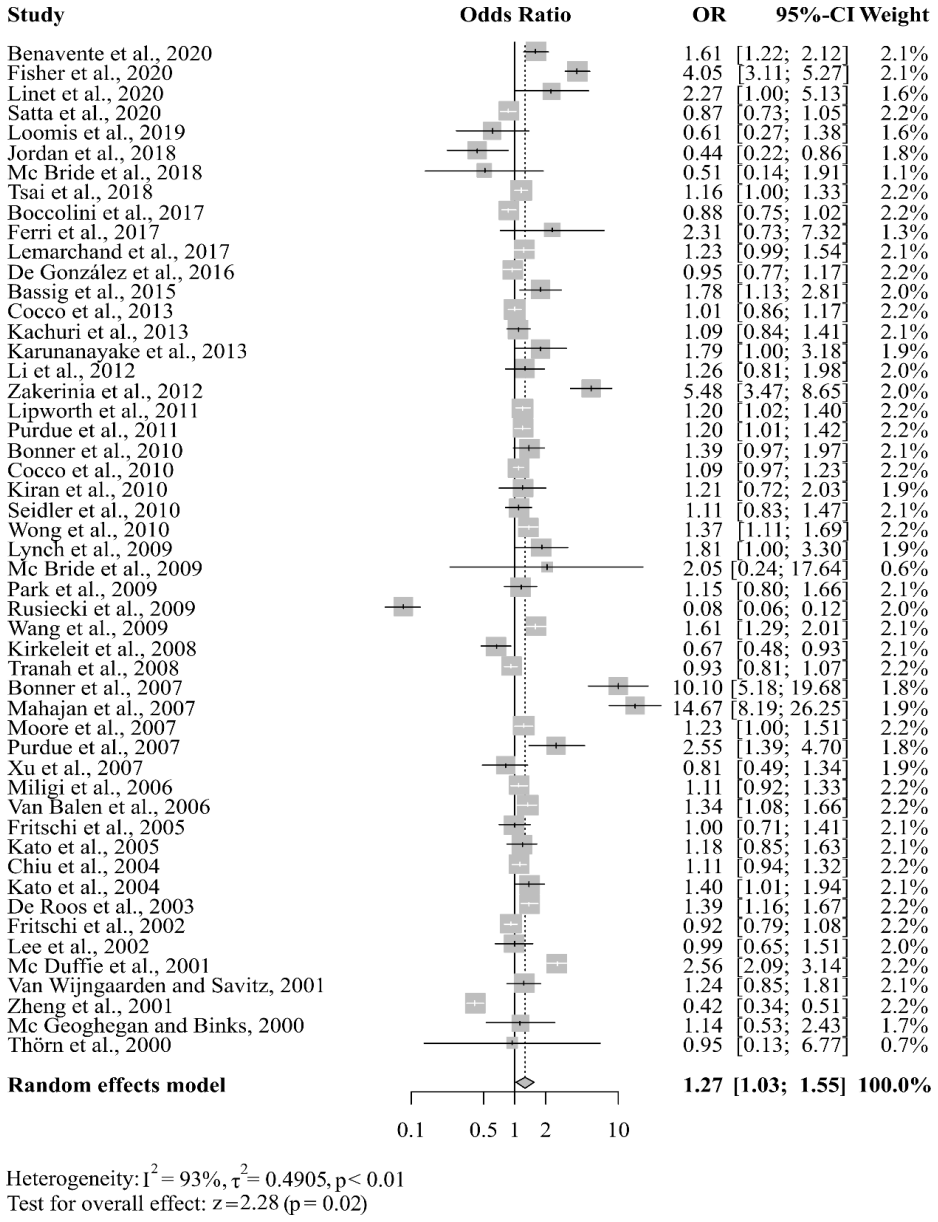

Figure 3. Forest plot of the overall odds ratio (OR) of random effects of occupational exposure and NHL risk [7–9,11,12,32,33,37–42,48–85].

Altogether, more than 20 different work classes and more than 30 chemical compounds were evaluated (Table 1). Of the 20 studies that exhibited significant OR, most (14 studies) evaluated individuals in the agricultural occupation, or occupationally exposed to pesticides. As for the carcinogenic potential of the compounds most evaluated in the articles that were included in the meta-analysis, pesticides present varied carcinogenicity classifi-

cations, given that the classification depends greatly on the active compound evaluated. The only pesticide analyzed in the articles that were included in the meta-analysis that is classified as carcinogenic for humans (Group 1), according to the International Agency for Research on Cancer [86], is Lindane, which exhibited sufficient evidence of carcinogenicity for non-Hodgkin lymphoma [50].

Although pesticides constitute the main exposure evaluated in the articles, four of the 20 articles that revealed significant OR had evaluated exposure to solvents, formaldehyde, trichloroethylene, and benzene, which were the main compounds analyzed in these articles. In addition, significant OR was also observed for studies that evaluated employees in the manufacture and operation of aircraft, employees exposed to meat, and factory workers exposed to benzene (Table 1). Formaldehyde, trichloroethylene, and benzene solvents are considered carcinogenic to humans according to the IARC classification based on the results of epidemiological studies [86].

Given the high heterogeneity observed in our meta-analysis, we carried out subgroup analyses as an approach to identify the potential sources of heterogeneity in our overall meta-effects estimate. Thus, combined estimates of studies by NHL subtype, experimental design types, and exposure types were conducted. In the analysis performed for each NHL subtype evaluated in more than three studies, included in our meta-analysis, a meta-OR above 1.0 with significance for the random effects model was only observed for the DLBCL (OR 1.13, 95% CI 1.04–1.23) (Figures 4A and S2).

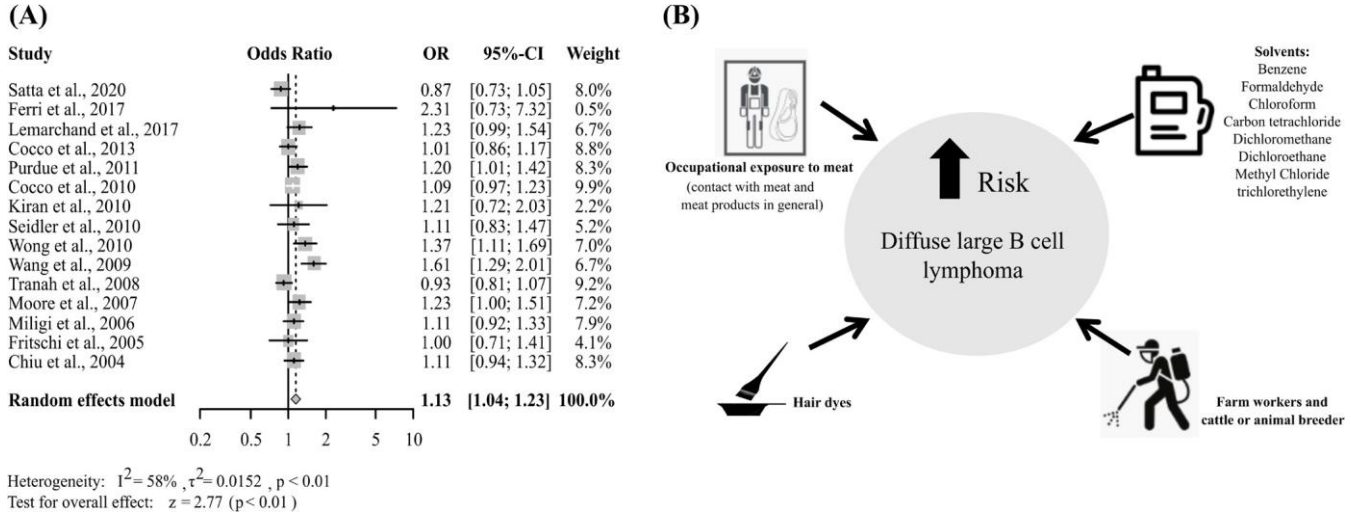

Figure 4. (**A**) Forest plot of the overall OR of occupational exposure and the risk of Diffuse Large B Cell Lymphoma; (**B**) Work classes and chemical compounds significantly associated with increased risk of Diffuse Large B Cell Lymphoma [8,9,49,55,56,63–66,68,70,71,73,74,76].

The most frequent subtype of non-Hodgkin lymphoma worldwide is DLBCL which presents as a heterogeneous disease group with variable outcomes [87]. In our meta-analysis, the individual studies that showed a significantly increased risk of DLBCL (OR > 1), analyzed populations exposed to solvents (benzene, formaldehyde, chloroform, carbon tetrachloride, dichloromethane, dichloroethane, and methyl chloride) [64,70] and hair dyes [66]. In addition, one study carried out their investigations with meat handlers, people in contact with meat and meat products in general (contact with beef, chicken, pork, lamb, meat from other animals and fish), [73] and another study with the agricultural occupations (farm workers and cattle or animal breeders) [66] (Figure 4B). Few meta-analysis studies separately assess the DLBCL subtype associated with any given type of exposure. One of the compounds evaluated by the articles that showed significant OR in our meta-analysis for the DLBCL subtype was benzene. Rana et al. [88] found an association between benzene exposure and an increased risk of DLBCL in their meta-analysis.

The remaining meta-analyses for the remaining subtypes generated meta-OR above the null value of 1.0, yet, with no significance (Figure S3). Some meta-analysis studies assessed the increased risk of NHL subtypes associated with exposures to chemical compounds. Similar to our study, increased risks of MM (OR = 1.16; 95% CI = 0.99–1.36) were detected in association with pesticide exposure [89] and trichloroethylene [90], however, without statistical significance. MM risk was also not significant for occupational exposure to polycyclic aromatic hydrocarbons [91]. On the other hand, in the meta-analysis by Chang and Delzell [92], they found significant meta-relative risks for the association between glyphosate and MM, however, for the subtypes DLBCL, LLC/SLL, FL, and Hairy Cell Leukemia, no significant association was observed.

A significant association between exposure to any single solvent and the risk of FL was also identified [21]. In our study, the meta-analysis for FL showed positive association (OR > 1) however, of no significance (OR = 1.17, CI: 0.95–1.44). We did observe a significant association for individual studies, including two studies that evaluated solvent exposure [63,68] (Figure S3C). Many studies have also demonstrated an association between benzene exposure and subtypes of NHL. Benzene exposure has been associated with an increased incidence of Cutaneous T Cell Lymphoma [93], and CLL [9].

When analyzing the different types of study designs, only the case-control studies analysis revealed a meta-OR above 1.0 with significance for the random-effects model (OR 1.21, 95% CI 1.03–1.41), while for the meta-analysis performed with the cohort studies, the meta-OR was less than 1.0 and non-significant (OR 0.82, 95% CI 0.36–1.92) (Figure S4). For the subgroup analyses by exposure type, three meta-analyses were performed, one considering studies that evaluated only work class, another evaluating only chemical compounds, and a third addressing only those articles that investigated both work class and exposure to chemical compounds (Figures 5–7).

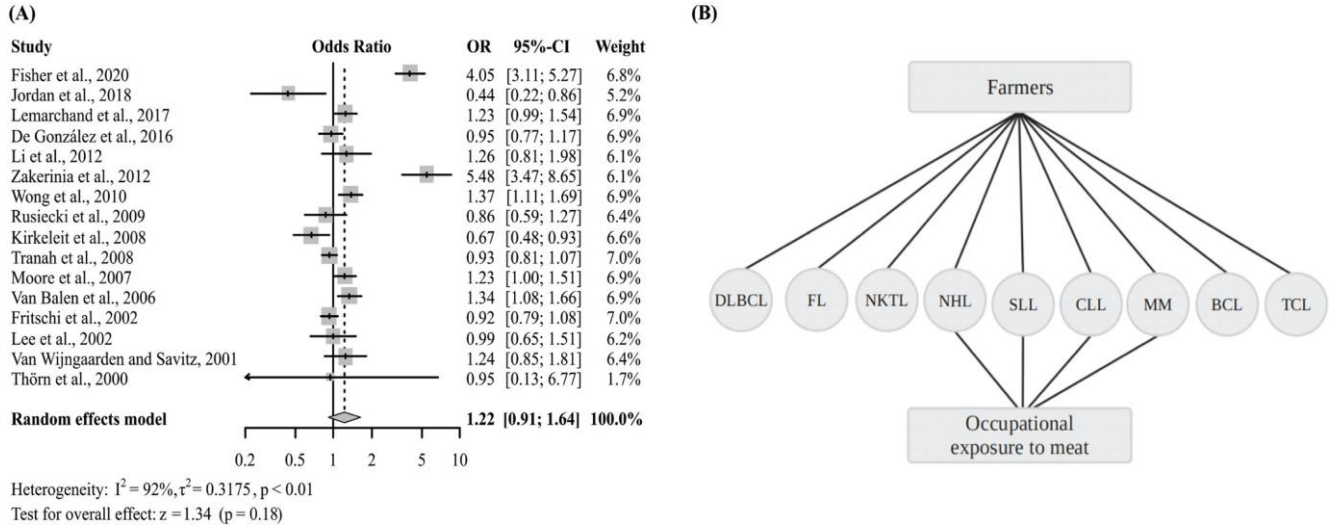

Figure 5. (**A**) Forest plot of the general OR of articles that only assessed work class and NHL risk; (**B**) Work classes significantly associated with increased risk of different subtypes of NHL. Diffuse Large B Cell Lymphoma (DLBCL), Follicular Lymphoma (FL), Natural killer/T-Cell Lymphoma (NKTL), Non-Hodgkin Lymphoma (NHL), Small Lymphocytic Lymphoma (SLL), Chronic Lymphocytic Leukemia (CLL), Multiple Myeloma (MM), B-cell Lymphomas (BCL), T-Cell Lymphoma (TCL) [11,12,41,42,51,56,57,61,66,69–71,79,80,82,85].

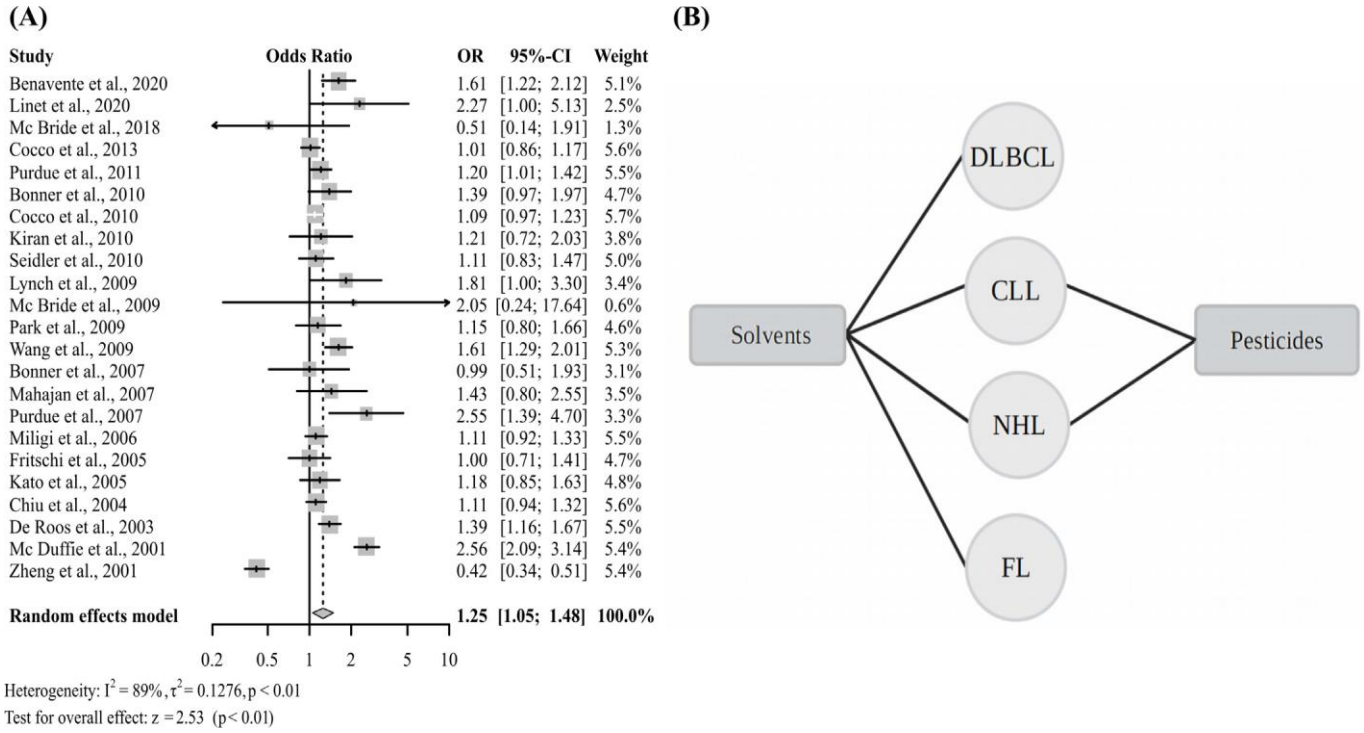

Figure 6. (**A**) Forest plot of the general OR of articles that evaluated only chemical compounds and the risk of NHL; (**B**) Chemical compounds significantly associated with increased risk of different subtypes of NHL. Diffuse large B Cell Lymphoma (DLBCL), Chronic Lymphocytic Leukemia (CLL), Non-Hodgkin Lymphoma (NHL), Follicular Lymphoma (FL) [7–9,32,33,37–40,48,52,63–65,67,68,73–76,78,81,83].

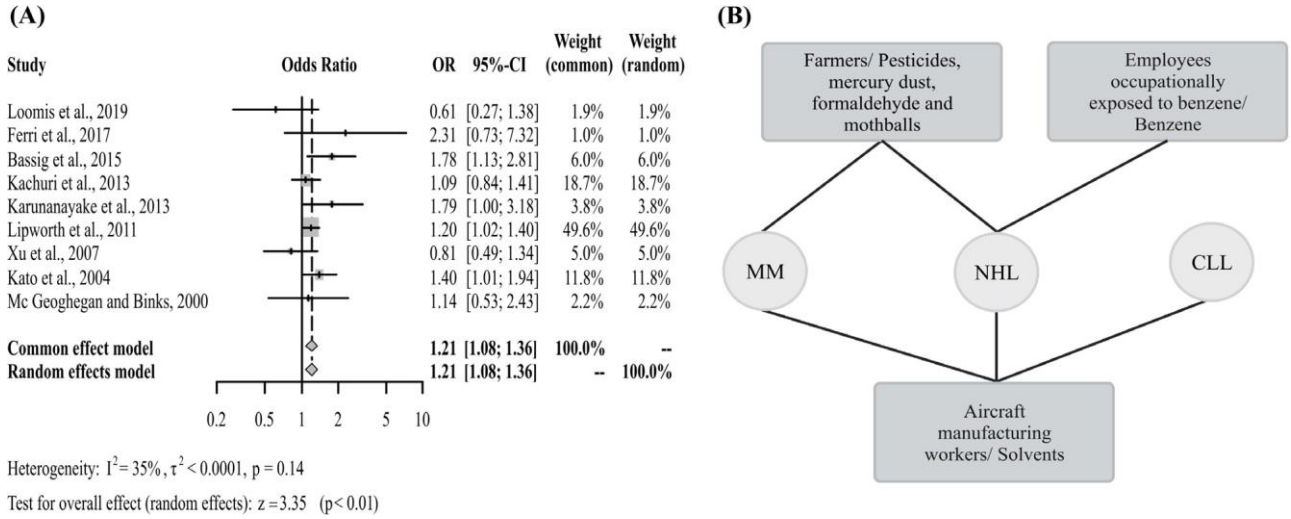

Figure 7. (**A**) Forest plot of the general OR of articles that evaluated work class and chemical compounds simultaneously and the risk of NHL; (**B**) Chemical compounds and work class significantly associated with increased risk of different NHL subtypes. Multiple Myeloma (MM), Non-Hodgkin Lymphoma (NHL), Chronic Lymphocytic Leukemia (CLL) [50,55,58–60,62,72,77,84].

A meta-analysis, which included studies that analyzed only work class, did not exhibit significantly increased risk of NHL (OR 1.22, 95% CI 0.91–1.64) (Figures 5A and S5). Despite not showing significant OR, five individual study results did show significant OR, among which, four assessed individuals in agricultural occupations [11,12,42,66] and one, of employees exposed to meat [71]. Furthermore, based on this analysis, NHL, B-Cell

Lymphoma, DLBCL, FL, T-Cell, MM, CLL, SLL, and T/NK Cell, were the subtypes of NHL addressed by the studies that presented a significantly increased risk of NHL. These results illustrate the potential risks posed by these two classes of work concerning the development of NHL regardless of subtype, however, further studies are needed (Figure 5B).

For the meta-analysis that included studies that examined only chemical compounds, significantly increased risk of NHL was observed (OR 1.24, 95% CI 1.06–1.46) (Figures 6A and S6). In this analysis, it was found that the studies with significant OR evaluated overall NHL, as well as the subtypes of DLBCL, FL, and CLL. Nine individual study results displayed significant OR, of which, five of the studies examined occupational exposure to pesticides [7,37,40,78,81]. A positive association between occupational exposure to some pesticides and the development of NHL was verified in the review study by Schinasi and Leon [94], in which they provided consistent evidence of this relationship. Exposure to glyphosate-based herbicides, 2,4-D and diazinon were also associated with increased risk of NHL in humans [22,46,47].

In addition to pesticide exposure, exposure to solvents, such as benzene (n = 1) [48], trichloroethylene (n = 1) [63], and solvents overall (n = 1) [68] (Figure 6B), was likewise observed in the studies. There is some evidence concerning the role of benzene in increasing the risk of NHL. In the meta-analysis studies by Rana et al. [88] and Steinmaus et al. [95], a causative link between benzene exposure and the development of NHL was evidenced. In contrast, the meta-analysis by Kane and Newton [96] found no association between benzene exposure and the increased risk of NHL or any subtype. Some studies have already demonstrated an association between exposure to trichloroethylene and the increased risk of NHL [63,93,97]. Our meta-analysis did not assess the individual association of compounds with the increased risk of NHL; however, we did find that these solvents were compounds evaluated in the studies that displayed a significant OR in our meta-analysis. The meta-analysis that included studies that examined both work class and chemical compounds did exhibit a significantly increased risk of NHL (OR 1.21, 95% CI 1.08–1.36) (Figures 7A and S7). The studies that showed significant OR in this analysis evaluated only the development of general NHL and/or MM and CLL subtypes. As for the exposure assessed in each of these studies, one of the studies looked at factory employees exposed to benzene along with the compound benzene [58]; another study evaluated individuals from the agricultural occupation, in addition to, pesticides, mercury, and formaldehyde [60]; there was another study with employees employed in aircraft manufacturing with exposure to chromate compounds, trichloroethylene, perchloroethylene, and mixed solvents [62]; and a further study included agricultural workers, and assessed exposure to pesticides and naphthalene [77] (Figure 7B).

Subgroup analyses such as NHL subtype, study design, and exposure type did not reveal the leading sources of heterogeneity observed in our overall meta-analysis (Figures 3 and S1). Nevertheless, considering the variety of NHL subtypes, the different possible settings for exposure (work class and chemical agents), exposure assessment methods, statistical results, study population, study site, and other factors evaluated in the studies included in our meta-analysis, we had already anticipated a high heterogeneity. In summary, despite the heterogeneity between studies in the different analyses performed and in our overall meta-analysis (Figure S1), we conclude that the synthesis of evidence from the epidemiological literature supports an increased risk for NHL. This result is independent of the subtype of NHL and the type of occupational exposure and compounds evaluated.

3.3. Strengths

Our meta-analysis synthesized 51 epidemiological studies examining the relationship between chemical agents and work classes and the risk of NHL. To the best of our knowledge, this is the first and largest systematic review with meta-analysis that takes into account all exposure types (chemical agents and/or work class) and all subtypes of NHL. Overall, our results provide evidence for the hypothesis that occupational exposure

to chemical agents increases the risk of NHL. Thus, these results represent an important contribution to the literature on exposures associated with the development of NHL.

3.4. Limitations

A limitation of the study is that our review was conducted with studies published between 2000 and 2020 (which included mature B-, T-, and NK-cell neoplasms as well as CLL and multiple myeloma as subtypes of LNH). However, according to the new classification of the World Health Organization to Hematolymphoid Tumors: Lymphoid Neoplasms (5th edition), the term LNH is no longer used [98]. The 51 studies that were reviewed in the analysis were quite heterogeneous, leading to high heterogeneity in the overall and subgroup meta-analysis. Although we examined possible sources of heterogeneity using factors such as NHL subtype, exposure type, and study design, there may be other possible causes of heterogeneity that could not be assessed. Adjustment for confounding factors such as anthropometric and sociodemographic variables was not performed, as most of the studies included had adjusted their risk measure for such factors. It is important to note that people are subject to both occupational and environmental exposure to a mixture of chemical compounds, causing the exposure profile to not be fully characterized in individual studies, and thus some of the observed associations may be due to chance. A further limitation of our study is that exposure, in most of the studies, was assessed by self-reported responses to epidemiologically applied questionnaires, which have the potential for memory bias and measurement error and not by objective and standardized measurements for exposure.

4. Conclusions

In this systematic review through meta-analysis, we present the evidence, through a detailed evaluation of epidemiologic studies, supporting the association between occupational chemical exposure and the risk of developing non-Hodgkin lymphoma. We demonstrate that the risk of NHL, regardless of the subtype, increases for individuals occupationally exposed to chemical agents, mainly pesticides, benzene, and trichloroethylene, as well as for certain work classes, primarily for occupations in agriculture. However, there is still insufficient data on the association between NHL and specific chemical compounds. Our findings may provide information for public health and practical decision-making about certain work activities and the use of chemical compounds. Furthermore, the evidence for the association of specific chemical compound classes and work classes associated with the development of NHL in biological samples is still limited, so future mechanistic studies, measuring exposures, and evaluating the biological and molecular effects associated with the risk of NHL are still needed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15092600/s1, Figure S1: Funnel Plot of the studies included in the overall meta-analysis. Each dot represents one study; Figure S2: Funnel Plot of the studies included in the meta-analysis for the Diffuse Large B Cell Lymphoma subtype. Each dot represents one study; Figure S3: Forest Plot and Funnel Plot of the studies included in the meta-analysis for (A) Multiple Myeloma subtype, (B) Chronic Lymphocytic Leukemia, (C) Follicular Lymphoma, and (D) B-Cell Non-Hodgkin's Lymphoma; Figure S4: Forest Plot and Funnel Plot of the (A) Case-control, and (B) Cohort and cross-sectional studies included in the meta-analysis; Figure S5: Funnel Plot of the studies included in the meta-analysis that evaluated only work class. Each point represents one study; Figure S6: Funnel Plot of the studies included in the meta-analysis that evaluated only chemical compounds. Each point represents one study; Figure S7: Funnel Plot of the studies included in the meta-analysis that simultaneously evaluated work class and exposure to chemical compounds. Each dot represents one study.

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4.2 Artigo 2- Analysis of the expression profile and biological function of plasma miRNAs in Chronic Lymphocytic Leukemia and Multiple Myeloma patients occupationally exposed to pesticides

ANALYSIS OF THE EXPRESSION PROFILE AND BIOLOGICAL FUNCTION OF PLASMA MIRNAS IN CHRONIC LYMPHOCYTIC LEUKEMIA AND MULTIPLE MYELOMA PATIENTS OCCUPATIONALLY EXPOSED TO PESTICIDES

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Abstract

Acute and chronic exposure to pesticides is associated with several negative effects on human health, such as increased risk of cancer, including mature B-cell neoplasms such as chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). However, the biological mechanism that links pesticide exposure and the development of CLL and MM has not yet been elucidated. Thus, this study aimed to explore miRNA expression profiles in patients with CLL and MM exposed to pesticides. For this purpose, 24 patients with MM and CLL were recruited from a cohort in Brazil and the miRNA expression profile between the groups was analyzed using Nanostring technology. The study revealed that the negative regulation of miR-423-3p, miR-1193, miR-576-5p, miR-509-5p, miR-548b-3p, miR-1469, miR-329-3p, and miR-548j-3p, and the positive regulation of miR-301b-5p and miR-548ah-5p in patients with CLL and MM were associated with occupational exposure to pesticides. The integrated network analysis between the differentially expressed miRNAs in the group of patients with exposure and their target genes demonstrated that miRNAs can participate in the regulation of signaling pathways, such as PI3K/Akt/mTOR and MAPK. Additionally, these miRNAs were found to be associated with an increased risk of developing neurological diseases and various types of cancer. In the present study, we demonstrate, for the first time, altered expression of plasma-derived miRNAs in patients with CLL and MM exposed to pesticides. The plasma miRNAs identified may serve as promising candidates for biomarkers of occupational exposure to pesticides, potentially contributing to the emerging field of Precision Environmental Health.

Keywords: Pesticides, chronic lymphocytic leukemia, multiple myeloma, miRNAs.

1. Introduction

In recent years, epidemiological studies have underscored the detrimental impact of pesticide exposure on human health. The increasing acute and chronic exposure to these substances, primarily due to occupational exposure, has demonstrated a significant risk to human health, as exposure to pesticides is linked to poisoning incidents and alterations in the nervous, digestive, circulatory, and respiratory systems (Kim et al., 2017; Mostafalou and Abdollahi, 2013; Payán-Rentería et al., 2012). Furthermore, studies have demonstrated a correlation between occupational exposure to pesticides and an elevated risk of specific types of cancer (Alavanja et al., 2014; Eriksson et al., 2008; Kim et al., 2017; Koutros et al., 2019; Lamure et al., 2019a; Lee et al., 2007; Martin et al., 2018; P. Sun et al., 2019; Ventura et al., 2019), including mature B-cell hematological neoplasms such as chronic lymphocytic leukemia (Alavanja et al., 2014; Benavente et al., 2020; Francisco et al., 2023; Leon et al., 2019) and multiple myeloma (Alavanja et al., 2014; Francisco et al., 2023; Louis et al., 2017; Rusiecki et al., 2009; Zakerinia et al., 2012).

Chronic lymphocytic leukemia (CLL) is the most prevalent form of leukemia in adults. It is a malignant disease characterized by the clonal expansion of mature CD5-positive B cells, which accumulate in the blood, bone marrow, lymph nodes, and spleen (Boelens et al., 2009; Crassini et al., 2019; Fabbri and Dalla-Favera, 2016; Linet et al., 2007; Rusiecki et al., 2009). In contrast, multiple myeloma (MM) is an incurable malignant disease of plasma cells that accumulate in the bone marrow, representing approximately 1% of all cancers (Tsang et al., 2019; van de Donk et al., 2021). The etiology of these diseases remains poorly understood, and the causes of the observed increase are largely unknown. Both diseases primarily affect individuals of advanced age. Genetic predisposition and variations, immunosuppression, infectious agents, environmental exposure, and exposure to chemical agents have been identified as risk factors associated with the development of CLL and MM (Slager et al., 2014; van de Donk et al., 2021).

A comprehensive collection of research has demonstrated a correlation between occupational exposure to a range of agricultural substances, including pesticides, fertilizers, and solvents, and an elevated risk of developing CLL and MM. This evidence is supported by findings from the Agricultural Health Study (AHS), a prospective study that included licensed applicators in Iowa and North Carolina (Alavanja et al., 2014,

Louis et al., 2017, Rusiecki et al., 2009, Bonner et al., 2010; Leon et al., 2019, De Roos et al., 2005, Rusiecki et al., 2004). Despite the existing evidence, the biological mechanism linking pesticide exposure and the development of CLL and MM remains to be elucidated.

It is established that pesticides can interact with genetic material, leading to genomic instability (Claudio et al., 2019; Kahl et al., 2018; Marcelino et al., 2019; Tomiazzi et al., 2018). This is regarded as the primary mechanism involved in carcinogenesis (Georgoulis et al., 2017; Pikor et al., 2013; Sabarwal et al., 2018). Nevertheless, epigenetic events that demonstrate an intrinsic link in the process of carcinogenesis (Hanly et al., 2018; Mostafalou and Abdollahi, 2013; Sabarwal et al., 2018; Silva and Jasiulionis, 2014; Ziech et al., 2010) remain under-explored in studies of occupational exposure to pesticides. Among epigenetic biomarkers, miRNAs represent a class of non-coding, single-stranded RNAs with approximately 22 nucleotides. They serve as post-transcriptional regulators of gene expression, inhibiting translation or degrading the target mRNA (Chen et al., 2019; Deng et al., 2019; Weldon et al., 2016). Given their stability at room temperature and presence in body fluids, miRNAs are regarded as prospective biomarkers in studies employing liquid biopsies, including the monitoring of occupational exposure and disease (Etheridge et al., 2011; Solé et al., 2018; Weber et al., 2010).

The potential of miRNAs to serve as biomarkers of pesticide exposure has been demonstrated in studies that have evaluated individuals with occupational exposure in comparison to those without such exposure (Gattuso et al., 2022; Weldon et al., 2016). Nevertheless, the relationship between altered miRNA expression profiles and occupational exposure to pesticides in patients with mature B-cell hematologic neoplasms has yet to be verified. Accordingly, the present study, within the context of precision environmental health, identified circulating miRNAs in the plasma of patients with CLL and MM exposed to pesticides as potential biomarkers of exposure. The potential biological function of the differentially expressed miRNAs was investigated through target prediction and enrichment analysis.

2. Methods

2.1 Patients Population

This was a prospective cross-sectional study of data collection and biological sampling. A total of 24 patients with mature B-cell hematologic malignancies were recruited from the Hematology Department of the Barretos Cancer Hospital, located in the city of Barretos, Brazil, between December 2019 and June 2022 (Figure 1). A simplified and expedient questionnaire, tailored to the specific type of cancer in question, was utilized to facilitate patient recruitment. Given that the patients treated at this hospital originate from a multitude of regions across Brazil, this questionnaire was devised to ascertain information pertaining to their occupations and potential exposure to carcinogens present in the workplace, which may contribute to the development of specific types of cancer (Vazquez et al., 2023). The data set comprised a range of variables, including age, body mass index (BMI), smoking status, alcohol consumption, and the patients' occupational and clinical histories. The data obtained from the questionnaires were stored in REDCap, a data management software (Wright, 2016).

The study included male and female patients, both occupationally exposed and unexposed to pesticides, diagnosed with Chronic Lymphocytic Leukemia/small cell lymphocytic lymphoma (n = 10) and Multiple Myeloma (n = 14). The inclusion criteria were as follows: patients with a confirmed diagnosis in accordance with the World Health Organization 2016 classification of lymphoid neoplasms (Swerdlow et al., 2016), aged over 18, who had not undergone any form of cancer treatment and had no history of previous cancer (Figure 1).

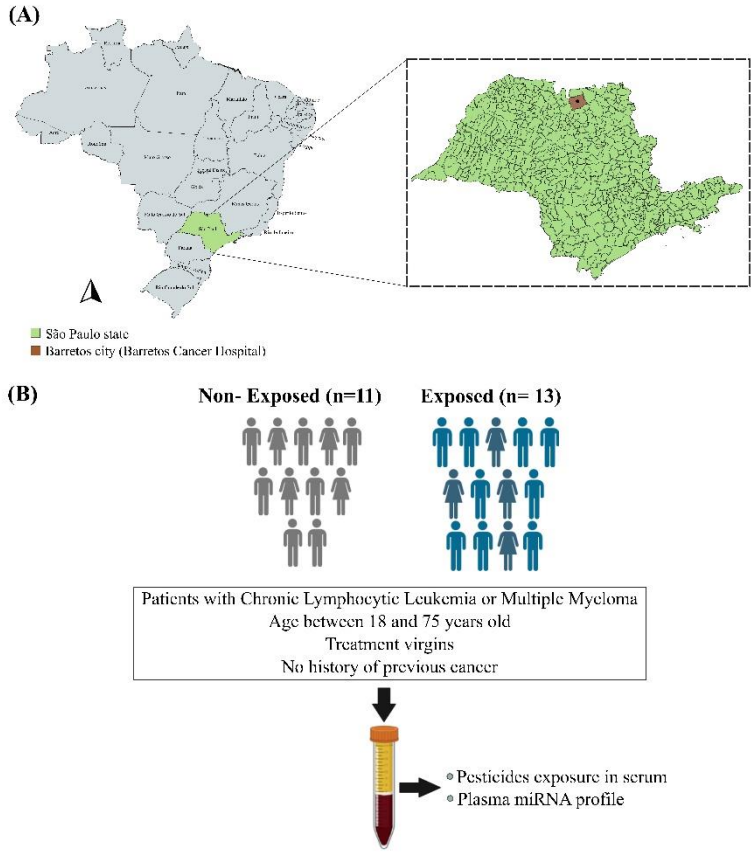


Figure 1. Study workflow: (A) Location of the study was the city of Barretos, located in the state of São Paulo, Brazil; (B) Eligible population and the analyses carried out in the study.

For patients meeting the eligibility criteria, peripheral venous blood was collected via vacuum tube and subsequently stored in the institution's biobank until the analyses were conducted (Neuber et al., 2022). The study was approved by the Institutional Ethics Committee of the Barretos Cancer Hospital (51644015.8.0000.5437). Prior to their participation in the study, all patients were required to sign a voluntary informed consent form.

2.2 Determination of pesticide exposure in serum

Analyses were performed by Gas Chromatography-Mass Spectrometry Tandem with an Agilent 7890 Gas Chromatograph coupled to a Waters Quattro Micro GC mass spectrometer employing a SLB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm). Aliquots of 400 µL of serum thawed at room temperature were prepared with organic solvents, internal standard, and sonication. Extraction was performed by Solid Phase Extraction with Waters Oasis Prime HLB 3cc (60 mg). Derivatization was performed with MTBSTFA and 1% TBDMSCl. The 23 determined compounds are

presented in supplementary material. Linearity presented as R2 were over 0.982 with LOQ between 0.3 and 5.6 $ng.mL^{-1}$. Values below the LOD were replaced with LOD divided by the square root of two (Hornung and Reed, 1990). Accuracy was determined intraday at 77-123%, whereas interday values were 69-124%. Precision was determined as coefficient of variation intraday 2-28% and interday 4-21%. This method was previously described (Birolli et al., 2024).

2.3 Measurement of miRNA level and normalization

The isolation of plasma miRNAs was conducted using the miRNeasy Serum/Plasma kit (Qiagen), in accordance with the manufacturer's instructions. The miRNA expression profile was analyzed using the Human v3 miRNA expression assay kit, in accordance with the instructions provided by the manufacturer (NanoString Technologies, Inc.). The analysis employs a unique fluorescent probe to directly digitalize the targets of interest, encompassing 798 distinct miRNAs.

In summary, the miRNA Preparation Protocol entailed a 1:500 dilution of the miRNA Assay Controls in DEPC H_2O, followed by the addition of 6.5 µL of this mixture to a combined ringing master mix comprising 13 µL of Ringing Buffer and 26 µL of nCounter miRNA Tag Reagent. For the annealing step, 3 µL of the sample (100 ng) was added to each tube. Following a 13-minute incubation period in a thermal cycler, a ligation master mix was prepared, comprising 19.5 µL of PEG and 13 µL of ligation buffer. Subsequently, 2.5 µL of the binding buffer was added to each tube, which was incubated in the thermal cycler for an additional five minutes at 48°C. During this incubation period, 1.0 µL of ligase was added to each tube. Immediately following the addition of ligase, the thermal cycler was closed, and the 24-minute ligation protocol was initiated. To perform the Purification Protocol, the tubes were removed from the thermal cycler and 1 µL of Ligation Clean-Up Enzyme was added to each tube. The tubes were then returned to the thermal cycler, where they were incubated for 1:10 minutes. Following the completion of the purification protocol, 40 µL of DEPC H_2O was added to each sample for denaturation at 85°C for a period of five minutes in the thermal cycler.

Subsequently, a final hybridization reaction was prepared, containing 10 µL of the Reporter CodeSet, 10 µL of hybridization buffer, a 5 µL aliquot of miRNA Preparation Protocol samples, and 5 µL of the Capture Probe Set. The Hybridization Protocol was conducted for a period of 24 hours, after which the hybridized miRNA-probe complexes

were captured on a streptavidin-coated surface and immobilized for subsequent enumeration. Digital images of the cartridges were obtained using the nCounter Digital Analyzer with a 550 FOV data resolution.

The miRNA count with the lowest coefficient of variation was employed for data normalization using the NanoStringNorm package in the R statistical environment (R-project, Vienna, Austria). A differential expression analysis was conducted using the Bioconductor "limma" package in the R environment (Ritchie et al., 2015) to identify statistically significant differences ($p < 0.05$) in miRNA expression between the groups exposed and unexposed to pesticides. To illustrate the miRNA expression profile, heatmaps were constructed using the ComplexHeatmap package (Gu et al., 2016), employing the fold change (FC) > 1 or < -1 to ascertain the positive or negative regulation of differentially expressed miRNAs.

2.4 Statistical analysis and bioinformatics

The samples were described using measures of frequency and proportion for the qualitative variables and mean and standard deviation for the quantitative variables. The comparison of these variables between the exposed and unexposed groups was conducted using Fisher's exact test and a two-sample t-test (parametric) or a Wilcoxon rank-sum test (non-parametric).

To identify the individual miRNAs associated with pesticide exposure, the miRNA count values obtained by the nSolver Analysis software version 2.6® (NanoString Technologies) were normalized according to the miRNA count with the lowest coefficient of variation for data normalization. This was achieved by using the NanoStringNorm package in the R statistical-mathematical environment (R-project, Vienna, Austria). A differential expression analysis was conducted using the Bioconductor "limma" package in the R environment (Ritchie et al., 2015) to identify statistically significant differences ($p < 0.05$) in miRNA expression between the pesticide-exposed and non-exposed groups. To illustrate the miRNA expression profile, heatmaps were constructed using the ComplexHeatmap package (Gu et al., 2016). The fold change (FC) value > 1 or < -1 was employed to ascertain the positive or negative regulation of differentially expressed miRNAs.

The predictive value of miRNAs as potential biomarkers of pesticide exposure was verified through the use of ROC curve analysis. The optimal cutoff value was

identified through a method that simultaneously maximizes sensitivity and specificity. The correlation between quantitative variables was calculated using Spearman's method, which is applicable in non-parametric contexts. Multivariate linear regression models were constructed with the 10 differentially expressed miRNAs as the response variable and the aforementioned covariates as the independent variable. These covariates included groups, Alpha Endosulfan, Beta Endosulfan, PBA, PhenylPyrazol, FipSul, gender, ethnicity, exposure time, staging (Durie Salmon), and staging subclass. The analysis employed the backward variable selection criterion, whereby the model commences with all independent variables and subsequently assesses the contribution of each to the prediction. Furthermore, principal component analysis (PCA) was employed to delineate the characteristics of the set of 10 miRNAs, and a graph of the first two principal components was constructed to illustrate their distinct features. For all analyses, a p-value of ≤ 0.05 was considered statistically significant.

2.5 miRNA target genes, gene enrichment and pathway analysis

To investigate the biological function of microRNAs (miRNAs) that are differentially expressed between groups, the top 10 miRNAs were selected based on the p-value. In silico putative target prediction was then performed using the miRNA data integration portal, mirDIP (**https://ophid.utoronto.ca/mirDIP/**). This portal was used because it allows the selection of only target genes with a strong interaction with miRNAs (top 1%, score class: very high). For the purposes of analysis, ten target gene prediction programs were considered, including DIANA, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and Targetscan. Only those targets that were present in at least five of the ten programs were subjected to further analysis. The miRNA target interaction network was constructed using the Cytoscape v3.9.1 software. To perform the functional analyses of the predicted target genes, the DAVID 6.8 Beta Knowledgebase (**https://david-d.ncifcrf.gov/**) database was employed for gene ontology (GO) analysis, which encompasses biological processes, cellular components, and molecular function, pathway enrichment analysis by the Kyoto Encyclopedia of Genes and Genomes (KEGG), and diseases associated with the target genes by the DisGeNET database. Only those terms with a p-value of < 0.05 were included.

3. Results

The sociodemographic, occupational, and clinical characteristics of the patients, including gender, ethnicity, age, BMI, smoking status, alcohol consumption, exposure time, and clinical staging, are presented in Table 1. A total of 24 patients were included in the study (16 men and 8 women), comprising 13 patients with occupational exposure to pesticides and 11 patients without occupational exposure. As illustrated in Table 1, the only statistically significant difference observed between the exposed and non-exposed groups pertains to smoking status. Specifically, the pesticide-exposed group exhibited a higher proportion of patients who had never smoked, whereas the non-exposed group demonstrated a higher proportion of ex-smokers. The group of occupationally exposed patients had been exposed to pesticides for approximately 20 years (Table 1).

Table 1. Characteristics of the study population.

Characteristics	**Exposed** (n = 13)	**Non-exposed** (n = 11)	**p-value**
Gender			
Male	9 (69%)	7 (64%)	> 0.9[1]
Female	4 (31%)	4 (36%)	
Ethnicity			
White	9 (69%)	6 (55%)	
Indigenous	2 (15%)	0 (0%)	0.20[1]
Brown	2 (15%)	5 (45%)	
Age (mean ± SD)	58.5 ± 7.82	62.8 ± 7.70	0.19[2]
BMI (mean ± SD)	27.3 ± 5.20	30.0 ± 8.15	0.37[2]
Smoking status			
Current	1 (7.7%)	1 (9.1%)	
In past	1 (7.7%)	7 (64%)	0.005[1]
Never	11 (85%)	3 (27%)	
Alcohol consumption			
No	11 (85%)	9 (82%)	
In past	1 (7.7%)	0 (0%)	0.80[1]
Yes	1 (7.7%)	2 (18%)	
Time of exposure to pesticides in years (mean ± SD)	20.5 ± 11.90	-	-
Stage*			
0	2 (15%)	1 (9.1%)	
I	0 (0%)	2 (18%)	
II	2 (15%)	2 (18%)	0.60[1]
III	8 (62%)	6 (55%)	
IV	1 (7.7%)	0 (0%)	
Subclass**			
A	9 (69%)	9 (82%)	0.60[1]
B	4 (31%)	2 (18%)	
ISS***			
I	3 (38%)	1 (20%)	0.50[1]

Table 1. Characteristics of the study population.

Characteristics	Exposed (n = 13)	Non-exposed (n = 11)	p-value
II	2 (25%)	3 (60%)	
III	3 (38%)	1 (20%)	

Note: SD: standard deviation; BMI: Body mass index.
[1]Fisher's exact test, [2] T-test.
* Durie-Salmon Staging System for MM and Rai and Binet Staging System for LLC.
** Subclass is based on the presence of symptoms B.
*** ISS: Multiple Myeloma International Staging System.

The quantified concentrations of pesticides in the serum of patients who have been occupationally exposed to pesticides and those who have not been exposed. No significant difference was observed in pesticide concentrations between the two groups, namely those occupationally exposed and those not exposed to pesticides (Table 2).

Table 2. Determination of pesticide in serum of occupationally exposed and non-exposed patients.

Pesticides	Occupationally Exposed ($ng.mL^{-1}$) [1]	Non-Occupationally Exposed ($ng.mL^{-1}$) [1]	p-value[2]
Dichlorodiphenyldichloroethylene (DDE)	4.6 (6.80)	5.0 (6.20)	> 0.9
Alpha-Endosulfan	1.21 (1.12)	0.60 (0.41)	0.2
Beta-Endosulfan	1.73 (1.69)	0.89 (1.01)	0.2
Gamma-HCH (Lindane) and Beta-HCH	3.31 (3.49)	2.39 (1.83)	0.8
4-Nitrophenol (PNP)	1.65 (0.73)	1.36 (0.47)	0.3
3-Phenoxybenzoic acid (PBA)	0.78 (0.11)	0.81 (0.15)	0.7
1-(4-Chlorophenyl)-1H-pyrazol-3-ol (PhenylPyrazol)	1.94 (1.68)	1.45 (1.63)	0.5
Fipronil sulfone (FipSul)	3.3 (4.50)	4.1 (3.80)	0.4
3,5,6-Trichloro-2-pyridinol (TCP)	0.84 (0.71)	1.04 (1.70)	0.6

[1]Mean (Standard Deviation).
[2]Wilcoxon rank sum exact test.

The analysis of the miRNA expression profile revealed seven differentially expressed miRNAs ($p \leq 0.01$) between the group of patients with CLL (n= 10) exposed and not exposed to pesticides (Figure 2A). In contrast, the analysis of the miRNA expression profile of patients with MM (n= 14) identified 22 differentially expressed miRNAs between the groups (Figure 2B). Subsequently, a pooled analysis of CLL and MM patients was conducted, and 32 differentially expressed miRNAs ($p \leq 0.05$) between the pesticide-exposed and non-exposed groups were identified, including 26 negatively regulated miRNAs and 6 positively regulated miRNAs (Figure 2C, Supplementary Table 2). The PCA analysis indicated a tendency for the patient samples to cluster by group, suggesting the existence of differential miRNA profiles between the pesticide-exposed and non-exposed groups (Figure 2D).

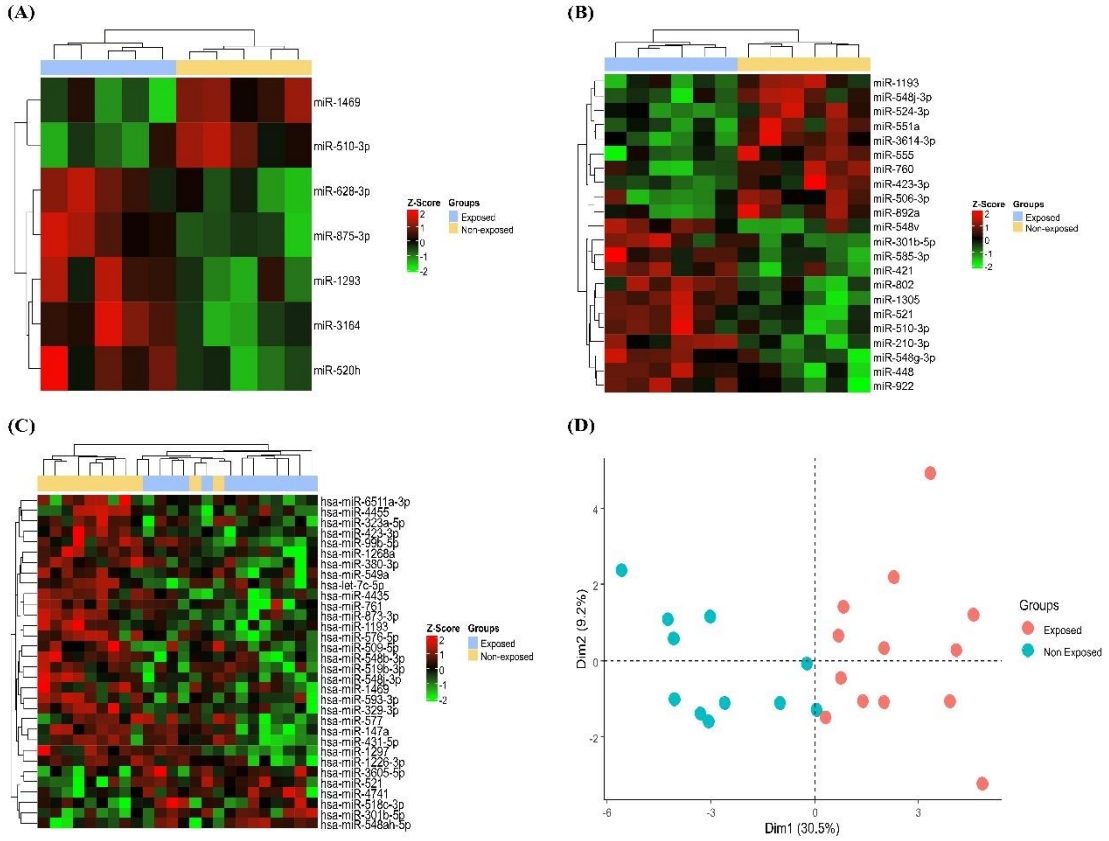


Figure 2. Heatmap of differentially expressed microRNAs (miRNAs) between pesticide-exposed and non-exposed groups using unsupervised hierarchical clustering for patients with chronic lymphocytic leukemia (CLL) ($p < 0.01$) (A); MM ($p < 0.01$) (B); and for the diseases collectively ($p < 0.05$) (C). The color of the dots represents the degree of expression: red dots represent positively regulated miRNAs, while green dots represent negatively regulated miRNAs. Principal component analysis (PCA) of plasma miRNAs between groups (D). The red dots represent patients exposed to pesticides, and the blue dots refer to samples from patients in the non-exposed group.

A comparison of the miRNAs shared between the two diseases revealed six miRNAs that met the significance threshold of $p \leq 0.05$ (Figure 3A). To ascertain the potential functions of the differentially expressed plasma miRNAs, the analysis was limited to the top 10 miRNAs based on the p-value ($p \leq 0.01$). Thus, two positively regulated miRNAs (miR-301b-5p and miR-548ah-5p) and eight negatively regulated miRNAs (miR-423-3p, miR-1193, miR-576-5p, miR-509-5p, miR-548b-3p, miR-1469, miR-329-3p, and miR-548j-3p) were observed in the group of patients exposed to pesticides (Figure 3B).

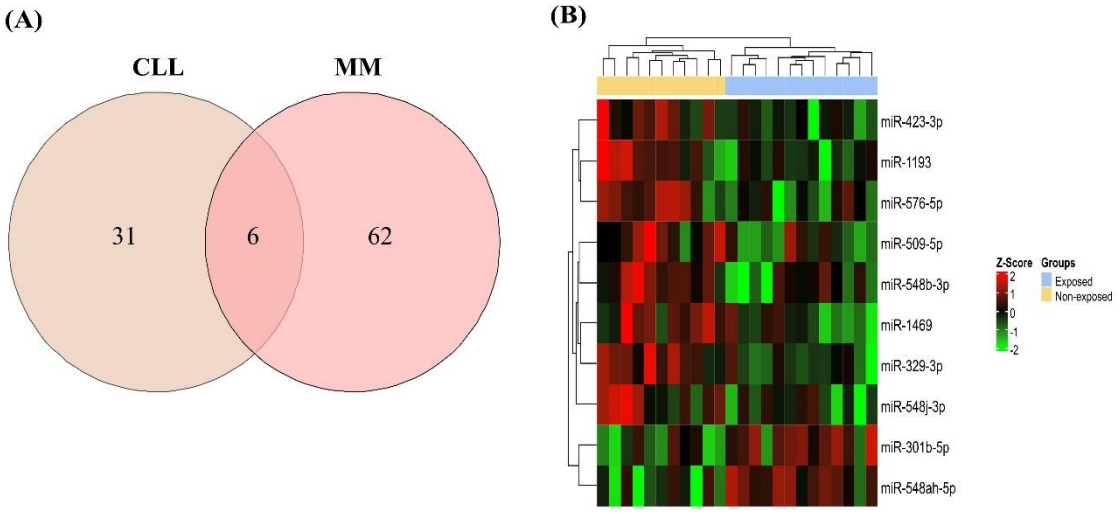
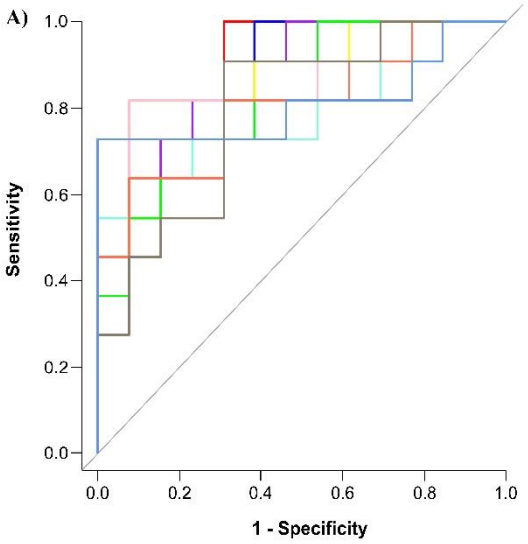


Figure 3. (A) Venn diagram showing the differentially expressed miRNAs superimposed between patients with CLL and MM; (B) Heatmap of the differentially expressed miRNAs between the pesticide-exposed and non-exposed groups with CLL and MM. The difference in miRNA expression between the two groups was assessed using the t-test ($p < 0.01$). The color represents the degree of expression: red represents positively regulated miRNAs, while green represents negatively regulated miRNAs.

To assess the predictive capacity of differentially expressed miRNAs as potential biomarkers of pesticide exposure, we employed receiver operating characteristic (ROC) curve analysis to ascertain the accuracy value (AUC). All of the differentially expressed miRNAs demonstrated an area under the curve (AUC) of ≥ 0.80 (Figure 4, Table 3).



B)

miRNAs	FC	p-valor	Specificity	Sensitivity	AUC
miR-329-3p	- 1.68	0.0007	0.69	1.0	0.90
miR-548ah-5p	1.35	0.0014	0.92	0.72	0.87
miR-1469	- 1.48	0.0023	0.84	0.72	0.84
miR-301b-5p	1.44	0.0029	0.69	0.90	0.85
miR-548b-3p	-1.83	0.0036	0.76	0.72	0.82
miR-576-5p	- 1.47	0.0038	0.92	0.81	0.85
miR-423-3p	- 1.90	0.0050	0.92	0.63	0.80
miR-548j-3p	- 1.46	0.0067	0.92	0.63	0.80
miR-509-5p	- 1.42	0.0071	0.69	0.90	0.80
miR-1193	- 1.46	0.0090	1.0	0.72	0.81

FC: fold-change; AUC: area under the curve.

Figure 4. (A) The receiver operating characteristic (ROC) curve of the ten differentially expressed miRNAs for prediction of potential biomarkers of pesticide exposure. Different colors indicate the AUC for each miRNA. (B) Predictive values of differentially expressed miRNAs in the group exposed to pesticides.

The correlation between the levels of plasma miRNAs that were differentially expressed in the exposed group and the variables of age, BMI, duration of exposure, and the concentrations of pesticides in serum are illustrated in Supplementary Figure 1. No significant correlation was observed between the variables BMI and age and any of the evaluated miRNAs. The results demonstrated a statistically significant inverse correlation between miR-548j-3p and 4-nitrophenol (R = -0.66, p-value = 0.01), miR-301b-5p and 3-phenoxybenzoic acid (R = -0.58, p-value = 0.04), and miR-548ah-5p and fipronil sulfone (R = -0.62, p-value = 0.03). Furthermore, the duration of exposure demonstrated a significant negative correlation with miR-576-5p (R = -0.62 and p-value = 0.02) and a significant positive correlation with miR-548ah-5p (R = 0.61 and p-value = 0.02) (Figure 5).

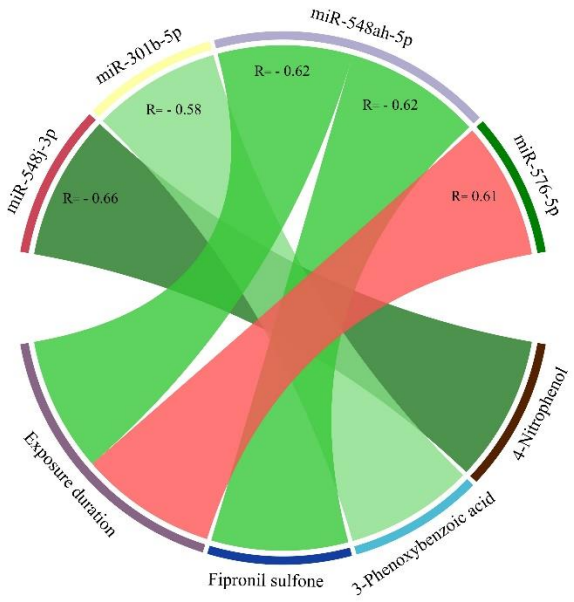


Figure 5. Significant Spearman correlation coefficients between miRNA expression levels and pesticide quantification in exposed individuals. The value of the correlation is indicated by the green and red colors, the green color represents a negative correlation, and the red color represents a positive correlation.

When analyzing the difference in expression of miRNAs in the group of patients with pesticide exposure, considering the variables gender, ethnicity, smoking, alcoholism and clinical staging, only a significant difference was observed for the levels of miR-576-5p in the variable gender, a difference in the levels of miR-548ah-5p for smoking status and miR-509-5p for the variable alcohol consumption (Supplementary Figure 2).

Subsequently, a multivariate linear regression analysis was conducted to assess the expression of miRNAs as a function of the evaluated predictor variables. The analysis revealed that the exposure variable was the primary predictor, exhibiting significant values in the models, except for miR-1193, miR-548b-3p, and miR-548j-3p. However, the pesticides beta-endosulfan and phenylpyrazol demonstrated significant correlations with the expression of miR-548b-3p, while alpha-endosulfan and 3-PBA correlated with miR-548j-3p (Table 4).

Table 4. Multivariate linear regression analysis between miRNAs expression levels and assessed variables.

Dependent variable	Independent variable	Adjusted R^2	β	p-value	95 % CI
miR-423-3p	Exposure	0.374	895.55	**0.001**	400.31 to 1390.78
	Smoking status		354.70	0.062	-20.55 to 729.95
miR-548ah-5p	Exposure	0.592	-191.85	**0.025**	-357.41 to -26.29
	Gender		-158.41	0.055	-321.06 to 4.23
	Smoking status		213.44	**0.002**	82.77 to (344.12
miR-1193	Exposure	0.252	257.34	0.052	-3.21 to 517.90
	Stage subclass		-258.17	0.079	-549.48 to 33.14
miR-548b-3p	Beta-Endosulfan	0.609	-168.65	**0.008**	-288.19 to -49.11
	PhenylPyrazol		-121.05	**0.022**	-222.49 to -19.60
	Gender		-351.03	0.052	-706.02 to 3.96
	Ethnicity		204.90	**0.043**	7.05 to 402.75
	Smoking status		-446.39	**0.003**	-721.04 to -171.75
	DS stage		-149.65	0.056	-303.89 to 4.58
miR-1469	Exposure	0.253	305.75	**0.009**	81.88 to 529.61
miR-576-5p	Exposure	0.420	339.30	**0.007**	103.39 to 575.20
	Gender		308.21	**0.016**	64.02 to 552.39
miR-548j-3p	Exposure	0.700	188.02	0.068	-15.99 to 392.04
	Alpha-Endosulfan		-196.58	**0.001**	-303.95 to -89.21
	3-PBA		1809.68	**0.0001**	1008.97 to 2610.39
	Ethnicity		141.83	**0.014**	32.70 to 250.96
	Age		-15.87	**0.012**	-27.77 to -3.97
miR-509-5p	Exposure	0.559	468.80	**0.0002**	250.17 to 687.43
	PhenylPyrazol		66.96	0.057	-2.41 to 136.33
	Gender		-266.81	**0.022**	-490.96 to -42.65
	Stage subclass		242.10	0.052	-3.19 to 487.40
miR-329-3p	Exposure	0.430	477.58	**0.003**	182.37 to 772.78
	PhenylPyrazol		-87.28	0.061	-179.28 to 4.70
miR-301b-5p	Exposure	0.439	-497.99	**0.0008**	-757.91 to -238.08
	Alpha-Endosulfan		-564.58	**0.011**	-985.68 to -143.49
	Beta- Endosulfan		294.62	**0.020**	50.85 to 538.38
	DS stage		-101.72	0.093	-222.44 to 19.00

DS stage: Durie-Salmon Staging System; 3-PBA: 3-Phenoxybenzoic acid.

In the target prediction analysis, the 10 miRNAs identified as differentially expressed between the groups were entered into a search for target genes. This was

because, in the multivariate linear regression analysis, variables of exposure or specific pesticides remained within the final model of the miRNAs of interest. The target gene prediction analysis yielded 1,217 target genes for the negatively regulated miRNAs (Figure 6A, Excel Table S1) and 159 target genes for the positively regulated miRNAs (Figure 6B, Excel Table S2).

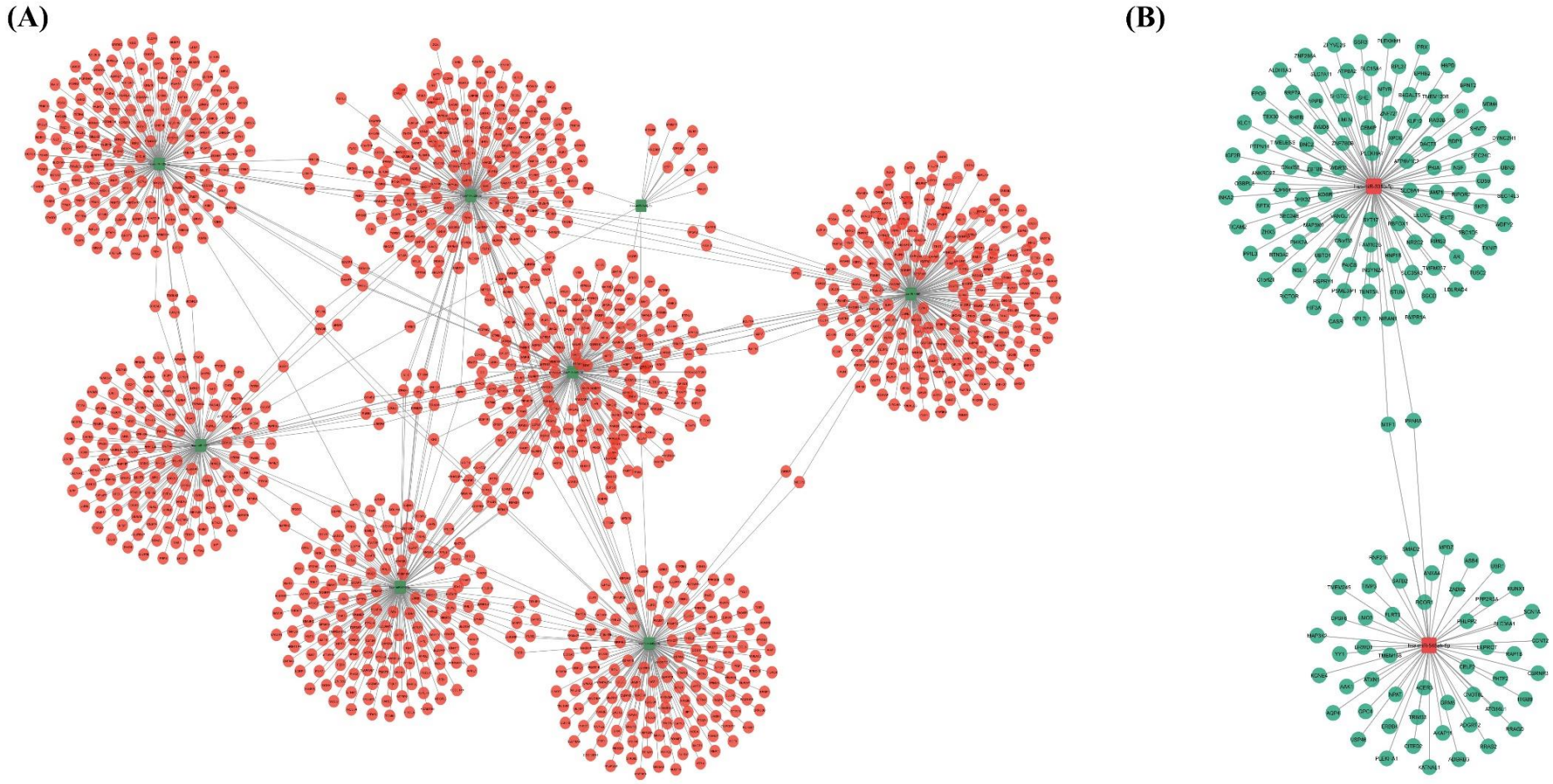
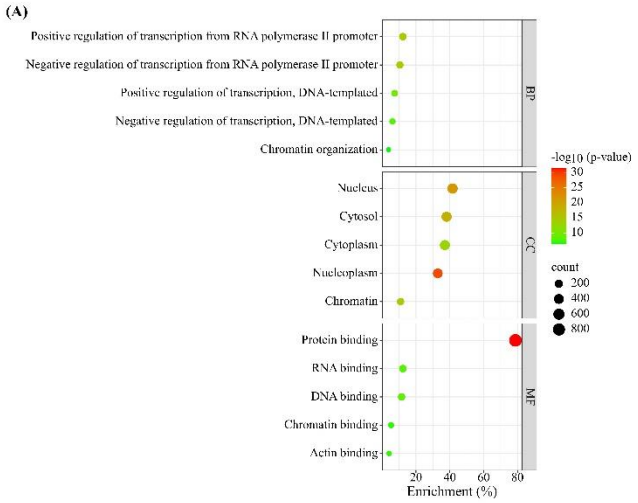
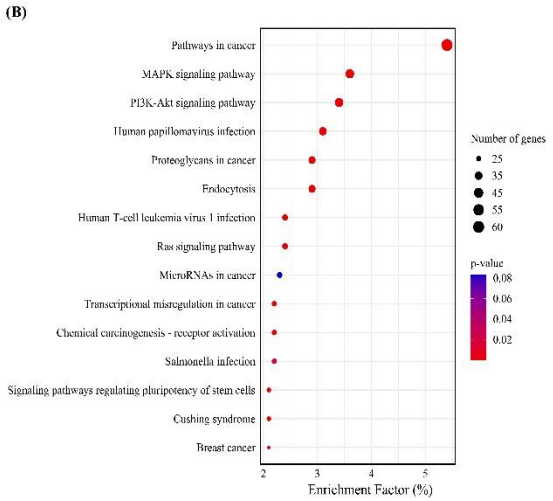


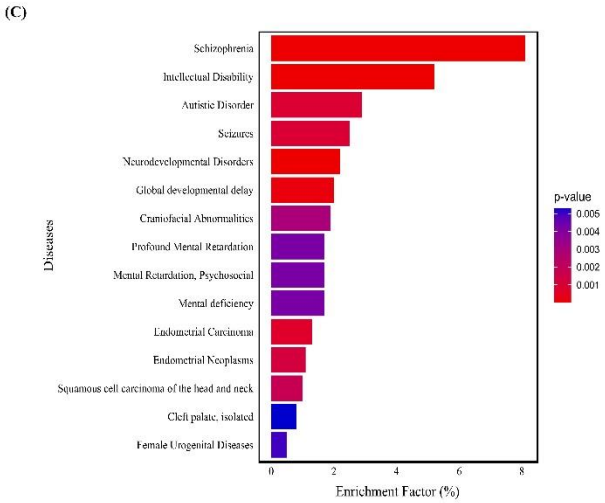
Figure 6. Interaction network between differentially expressed miRNAs and their validated target genes; (A) Interaction network between negatively expressed miRNAs and their target genes. Red circles represent target genes and green circles represent negatively regulated miRNAs; (B) Interaction network between positively expressed miRNAs and their target genes. The green circles represent target genes, and the red circles represent the positively regulated miRNAs.

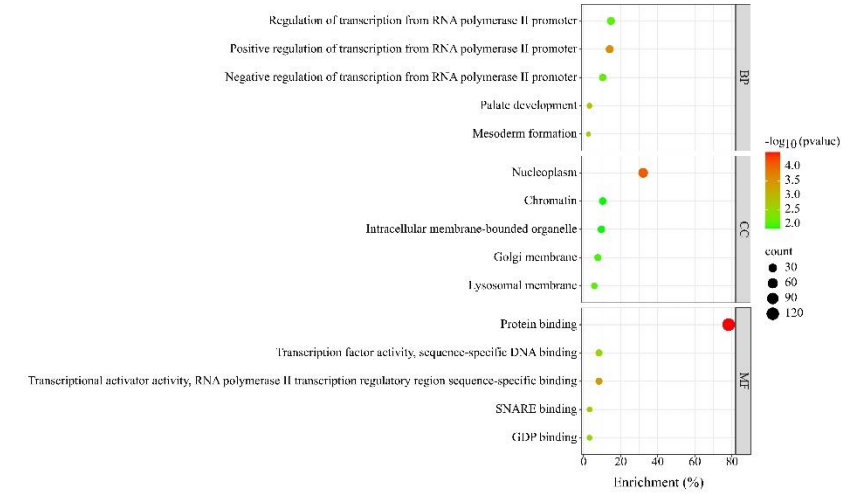
A functional enrichment analysis of the target genes of the negatively regulated miRNAs revealed the presence of 302 biological processes, 104 cellular component terms, and 97 molecular function terms (Figure 7A, Excel Table S3, S4, and S5). Furthermore, 77 enriched pathways were found to be significantly associated with the target genes of the negatively regulated miRNAs (Figure 7B, Excel Table S6). The most significantly enriched pathways were those related to various types of cancer. The target genes of the negatively regulated miRNAs were also found to be associated with 76 diseases (Figure 7C, Excel Table S7). With regard to the significantly annotated diseases, the majority were diseases associated with the neurological system and cancer (Figure 7C, Excel Table S7).

In relation to the predicted targets of the positively regulated miRNAs, 28 biological processes, 12 cellular component terms, and 13 molecular function terms were found to be significantly associated with the target genes (Figure 7D, Excel Table S8, S9, and S10). Upon analysis of the predicted pathways of the positively regulated miRNAs target genes, only the mTOR signaling pathway was found to be significantly associated ($p= 0.047$) with the predicted target genes (Excel Table S11). With regard to the diseases related to the predicted targets of the positively regulated miRNAs, three diseases were identified with a significant association (Figure 7E, Excel Table S12).


(A)
Positive regulation of transcription from RNA polymerase II promoter
Negative regulation of transcription from RNA polymerase II promoter
Positive regulation of transcription, DNA-templated
Negative regulation of transcription, DNA-templated
Chromatin organization
Nucleus
Cytosol
Cytoplasm
Nucleoplasm
Chromatin
Protein binding
RNA binding
DNA binding
Chromatin binding
Actin binding
BP
CC
MF
20
40
60
80
Enrichment (%)
-log10 (p-value)
30
25
20
15
10
count
200
400
600
800


(B)
Pathways in cancer
MAPK signaling pathway
PI3K-Akt signaling pathway
Human papillomavirus infection
Proteoglycans in cancer
Endocytosis
Human T-cell leukemia virus 1 infection
Ras signaling pathway
MicroRNAs in cancer
Transcriptional misregulation in cancer
Chemical carcinogenesis - receptor activation
Salmonella infection
Signaling pathways regulating pluripotency of stem cells
Cushing syndrome
Breast cancer
2
3
4
5
Enrichment Factor (%)
Number of genes
25
35
45
55
60
p-value
0.08
0.06
0.04
0.02


(C)
Schizophrenia
Intellectual Disability
Autistic Disorder
Seizures
Neurodevelopmental Disorders
Global developmental delay
Craniofacial Abnormalities
Profound Mental Retardation
Mental Retardation, Psychosocial
Mental deficiency
Endometrial Carcinoma
Endometrial Neoplasms
Squamous cell carcinoma of the head and neck
Cleft palate, isolated
Female Urogenital Diseases
Diseases
0
2
4
6
8
Enrichment Factor (%)
p-value
0.005
0.004
0.003
0.002
0.001


(D)
Regulation of transcription from RNA polymerase II promoter
Positive regulation of transcription from RNA polymerase II promoter
Negative regulation of transcription from RNA polymerase II promoter
Palate development
Mesoderm formation
Nucleoplasm
Chromatin
Intracellular membrane-bounded organelle
Golgi membrane
Lysosomal membrane
Protein binding
Transcription factor activity, sequence-specific DNA binding
Transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding
SNARE binding
GDP binding
BP
CC
MF
0
20
40
60
80
Enrichment (%)
-log10 (pvalue)
4.0
3.5
3.0
2.5
2.0
count
30
60
90
120

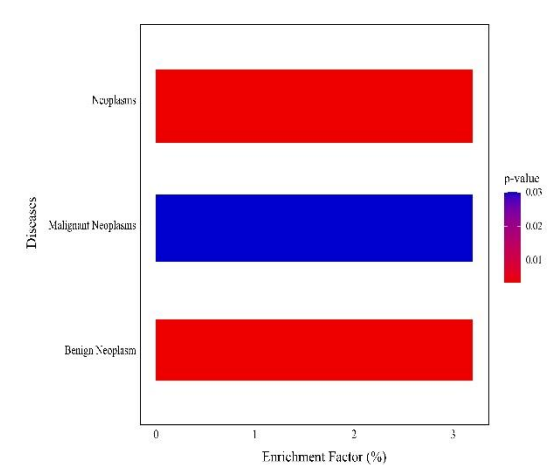

(E)
Neoplasms
Malignant Neoplasms
Benign Neoplasm
Diseases
0
1
2
3
Enrichment Factor (%)
p-value
0.03
0.02
0.01

Figure 7. Functional annotation of validated target genes for differentially expressed miRNAs. (A) TOP 5 Gene ontology term of three categories for the targets of negatively regulated miRNAs. All terms have a p-value less than 0.05. The colors of the dots represent the p-value and the sizes of the dots the number of genes associated with each term; (B) Top 15 Pathway based on the KEGG pathway database for the targets of negatively regulated miRNAs. All terms have a p-value less than 0.05. The colors of the dots represent the p-value and the sizes of the dots the number of genes associated in each pathway; (C) Top 15 diseases from the DisGeNET database associated with the predicted target genes for the negatively regulated miRNAs. The colors of the bars represent the p-value; (D) TOP 5 Gene ontology terms of three categories for the targets of the up-regulated miRNAs. All terms have a p-value less than 0.05. The colors of the dots represent the p-value and the sizes of the dots represent the number of genes associated with each term; (E) Diseases from the DisGeNET database associated with the predicted target genes for positively regulated miRNAs.

4. Discussion

In the present study, we demonstrate, for the first time, altered expression of plasma-derived miRNAs in patients with CLL and MM exposed to pesticides. The serum levels of the quantified pesticides were found to be generally higher in patients occupationally exposed to pesticides than in non-exposed patients, with the exception of DDE, FipSul, PBA, and TCP. The presence of pesticide residues in the general population at concentrations of a few ng mL^{-1} has been observed in numerous studies conducted in all regions of the world. DDE, quantified at approximately 5.0 ng mL^{-1} in this study, and its precursors have been detected at concentrations of 0.9, 8.4, 3.4, and 0.9 ng mL^{-1} in various countries, including Norway (Rylander et al., 2012), Mexico (Waliszewski et al., 2012), China (Miao et al., 2021), and the USA (United States National Health and Nutrition Examination Survey, 2020), respectively. The age of the patients, which is approximately 60 years, is a relevant factor for the quantification of this compound in our study. This is because DDE is a persistent and bioaccumulative compound, and it is often correlated with the age group of the participants (Wang et al., 2017). Similarly, the active ingredient gamma-HCH was detected in the form of a legacy insecticide, with concentrations of 3.3 ng mL^{-1} for the occupationally exposed group and 2.4 ng mL^{-1} for the control group. It is noteworthy that concentrations of this magnitude have been documented in other countries, with values of 2.7 ng mL^{-1} in Mexico (Waliszewski et al., 2012) and 2.0 ng mL^{-1} in China (Miao et al., 2021), as well as in other regions within Brazil (Freire et al., 2013).

Endosulfan was banned from use in Brazil in 2010 (Brazil, 2010), thus its application has a more recent history. Concentrations of 1.2 and 1.7 ng mL^{-1} for alpha- and beta-endosulfan, respectively, were observed in the occupationally exposed group. In contrast, the non-exposed group exhibited concentrations of 0.6 and 0.8 ng mL^{-1},

respectively. Additionally, endosulfan has been identified as a legacy pesticide in other countries, including Wuhan in China, where a concentration of 1.2 mg mL^{-1} for alpha-endosulfan was determined (Miao et al., 2021). However, other studies have reported lower concentrations of this pesticide (Freire et al., 2013; González-Alzaga et al., 2018).

The presence of pesticide residues, including 4-nitrophenol, 3-phenoxybenzoic acid, and 3,5,6-trichloro-2-pyridinol, was observed at concentrations of 1.7, 0.8, and 0.8 ng mL in serum, respectively. Typically, these active ingredients are determined in urine, as evidenced by the concentrations reported in the United States National Health and Nutrition Examination Survey. The survey described average concentrations of 1.0 ng mL^{-1} for 4-nitrophenol, 1.8 ng mL^{-1} for 3,5,6-trichloro-2-pyridinol, and 2.0 ng mL^{-1} for 3-phenoxybenzoic acid (United States National Health and Nutrition Examination Survey, 2023). Fipronil is an insecticide that is widely used in tropical climates to combat ants and other pests (Avicor et al., 2023). This may explain the determination in this study of concentrations of 3.3 ng mL of fipronil sulfone for occupationally exposed patients and 4.1 ng mL^{-1} for the non-exposed group. Furthermore, studies in the literature indicate that this compound is frequently detected, as evidenced by the analysis of adults from the city of Wuhan (China), which revealed concentrations ranging from 0.1 to 1.5 ng mL in serum (Shi et al., 2021).

At present, there is a noticeable absence of human biomonitoring of the fungicide Pyraclostrobin, largely due to the absence of a widely accepted chemical biomarker of exposure in the scientific literature (Leite et al., 2024). In this study, the potential use of 1-(4-chlorophenyl)-1H-pyrazol-3-ol as an exposure biomarker was proposed, given that this compound represents a primary metabolite of the active ingredient in both animal and environmental samples (Birolli et al., 2020; Yoshizawa et al., 2018). Furthermore, it was detected in this study at an average concentration of 0.5 ng mL^{-1}.

The pervasive use and increased exposure of humans to pesticides have prompted a growing interest in the biological effects of such exposure on human health. Some epidemiological studies have demonstrated an association between exposure to pesticides and the development of mature B-cell neoplasms, including CLL and MM (Benavente et al., 2020; Kachuri et al., 2013; Lemarchand et al., 2017; Zakerinia et al., 2012). Additionally, Francisco et al. (2023) conducted a meta-analysis, which indicated that exposure to pesticides is associated with an elevated risk of developing CLL and MM, as well as other types of mature B-cell neoplasms. Lamure et al. (2019) demonstrated that

patients with diffuse large B-cell lymphoma who had been exposed to pesticides exhibited a diminished event-free survival rate at two years when compared to patients who had not been exposed. Despite these results and the epidemiological studies conducted to date, the molecular mechanisms by which pesticide exposure may contribute to carcinogenesis remain incompletely understood. Accordingly, the present study employed miRNA expression profiling to gain deeper insight into the epigenetic mechanisms associated with pesticide exposure in patients with CLL and MM. The analyses demonstrated decreased expression of eight miRNAs (miR-423-3p, miR-1193, miR-576-5p, miR-509-5p, miR-548b-3p, miR-1469, miR-329-3p and miR-548j-3p) and increased expression of two miRNAs (miR-301b-5p and miR-548ah-5p) in patients exposed to pesticides when compared to patients without exposure.

The results of the correlation analysis indicated that an elevated quantification of the pesticides FipSul, 4-Nitrophenol, and Phenoxybenzoic acid was inversely associated with the expression of miR-548ah-5p, miR-548j-3p, and miR-301b-5p, respectively. Furthermore, the duration of exposure to pesticides demonstrated a negative correlation with miR-576-5p and a positive correlation with miR-548ah-5p. In this study, a series of multiple regression models were constructed to investigate the potential associations between miRNAs and the variables that may influence their expression. The analysis demonstrated that certain confounding factors, including smoking status, gender, ethnicity, and age, exerted a significant influence on the expression of specific miRNAs. Conversely, all miRNAs exhibited a notable association with the exposure factor or an individual pesticide. To the best of our knowledge, no previous study has investigated the potential association between exposure to specific pesticides or the overall exposure factor and the differential expression of miRNAs in patients with CLL and MM.

MicroRNAs (miRNAs) are a class of non-coding RNA that are considered to be important post-transcriptional regulators of gene expression. They are involved in a number of cellular processes and pathways. In the present study, we identified 1,124 predicted target genes for the eight negatively regulated miRNAs associated with occupational exposure to pesticides. The results of the enrichment analysis indicated that the target genes are associated with a number of biological processes, cellular components, molecular functions, and pathways. The three main pathways identified as being shared by several target genes of the negatively expressed miRNAs were found to be the Pathways in Cancer, the MAPK Signaling Pathway, and the PI3K-Akt Signaling

Pathway. The PI3K/Akt/mTOR and MAPK signaling pathways are of great significance for numerous biological and cellular processes, including cell growth, metastasis, survival, metabolism, migration, and apoptosis (Laplante and Sabatini, 2012; Shi et al., 2024; Yu et al., 2022). These pathways are deregulated in certain types of cancer, as they are implicated in the initiation, promotion, and progression of tumors.

The PI3K/Akt/mTOR pathway is known to be deregulated in lymphomas, including primary central nervous system lymphoma. Consequently, this pathway has emerged as a potential therapeutic target for the treatment of lymphomas (Majchrzak et al., 2014; Zhang et al., 2022). In light of these considerations, the specific PI3Kδ inhibitor idelalisib (trade name Zydelig) was approved by the FDA for the treatment of relapse in cases of CLL, non-Hodgkin's lymphoma, and small cell lymphocytic lymphoma (Markham, 2014). Alterations in the mitogen-activated protein kinase (MAPK) pathway (Ras/RAF/MEK/ERK pathway) have been demonstrated to be associated with certain types of hematological tumors. Additionally, studies have suggested the potential of this pathway as a therapeutic target for specific types of lymphoma (Ding et al., 2009; Elenitoba-Johnson et al., 2003; Huang et al., 2019; Vega et al., 2015; Wang et al., 2018).

In relation to the two microRNAs that were identified as being positively regulated in our study, a total of 158 predicted target genes were identified. These genes are involved in several different biological processes, as well as a variety of cellular components and molecular functions. However, in relation to the predicted pathways, only the mTOR signaling pathway was identified as being significantly enriched for target genes. The mTOR signaling pathway is associated with a number of processes, including growth, cell proliferation, synaptic plasticity, and the regulation of homeostasis in response to environmental signals (Laplante and Sabatini, 2012; Meng et al., 2013). Moreover, the mTOR signaling pathway is linked to a range of pathological conditions, including type 2 diabetes, neurodegenerative diseases, and cancer (Laplante and Sabatini, 2012), including the findings of Zhang et al. (2022) who demonstrated that the pathway's aberrant activation in patients with primary central nervous system lymphoma can result in disease recurrence and a reduction in progression-free survival.

The study demonstrated that the target genes of negatively expressed miRNAs are associated with a range of diseases, predominantly neurological disorders and various forms of cancer. As evidenced by prior studies, miR-423-3p expression is altered in patients with colorectal cancer (Li et al., 2015), gastric cancer (Kong et al., 2017), lung

cancer (Zhu et al., 2017), and prostate cancer (Ku et al., 2021). Alterations in miR-1193 have been observed in patients with T-cell leukemia (Shen et al., 2017), cutaneous squamous cell carcinoma (Lu et al., 2021), prostate cancer (Wang et al., 2021), intraepithelial lesions and cervical cancer (Zhang et al., 2020). Alterations in miR-576-5p was altered in patients with colorectal cancer (Zhou et al., 2021), endometrial cancer (Chen et al., 2023), ovarian cancer (Wu et al., 2022), papillary thyroid carcinoma (Hai et al., 2022), and triple negative breast cancer (Hadavi et al., 2019).

Some studies have demonstrated that the expression of miR-509-5p is altered in patients with pancreatic cancer (Li et al., 2017), non-small cell lung cancer (Wang et al., 2017) and renal cell carcinoma (Zhang et al., 2013). Additionally, Sun et al. (2019) reported that altered expression of miR-509-5p is associated with male infertility and testicular germ cell tumors. Conversely, miR-548b-3p was demonstrated to be negatively regulated in tissues from patients with hepatocarcinoma (Lin and Wang, 2018), colorectal cancer (Duan and Qiu, 2022) and lung cancer (Wang et al., 2020). The expression of miR-1469 was altered in tissues from patients with esophageal squamous cell cancer (Liu et al., 2017) and melanoma (DiVincenzo et al., 2021). miR-329-3p was found to be negatively regulated in tissues from patients with hepatocarcinoma (Xin et al., 2020), gastric cancer (Kan et al., 2023), colorectal cancer (Liu et al., 2020) and endometrial carcinoma (Wang et al., 2023). miR-548j-3p was observed to undergo alteration in patients diagnosed with peripheral arterial disease (Lee et al., 2022), chronic hepatitis B (Yu et al., 2017), and breast tumors with lymph node metastases (Zhan et al., 2016).

In regard to miR-301b-5p and miR-548ah-5p, which were identified in our study with increased expression in the group of patients exposed to pesticides, we have identified several studies that have reported altered miR-301b expression in prostate cancer (Fort et al., 2018) and colon cancer (Wang et al., 2010). With regard to miR-548ah-5p, Xing et al. (2014) demonstrated in their study the regulation of miR-548ah-5p in the different stages of chronic hepatitis B.

It is important to acknowledge the limitations of this study. The relatively modest sample size of the study limits the statistical power of the findings. Secondly, the miRNAs identified as differentially expressed in the pesticide-exposed group were not subjected to validation. Accordingly, the altered expression of these miRNAs must be verified in a larger patient population to confirm that they are differentially expressed in patients with mature B neoplasms exposed to pesticides. Nevertheless, we were able to ascertain

experimentally validated genes that are targets of the miRNAs identified in our study through the databases, as well as predict their potential biological functions and enriched pathways. Third, despite our efforts to control for the basic individual characteristics, lifestyle, and environmental and occupational exposure of the patients included in the study, the results of the associations between individual pesticides and miRNAs may have been influenced by individual lifestyle factors, as well as other pollutants that require further analysis.

Additionally, several strengths were identified in this study. The patients were recruited from diverse geographical regions within the country, which demonstrated that the miRNA expression profile identified in the study is directly associated with exposure. The relationship was confirmed through multivariate analysis, which demonstrated that the exposure factor was present in all the final regression models. Another advantage was the use of Nanostring technology, which is highly sensitive, and a panel comprising numerous target miRNAs to identify the miRNAs that were differentially expressed in the group of patients with pesticide exposure. Furthermore, we employed liquid biopsy (plasma) to investigate, for the first time in a comprehensive manner, the impact of pesticide exposure on the miRNA profile (epigenetic mechanisms) of CLL and MM patients. The results demonstrated that the miRNAs differentially expressed in the exposed patient group have the potential to regulate a number of genes that have been linked to a range of diseases, including cancer and neurological disorders, which have previously been identified as being associated with pesticide exposure in epidemiological studies. It is thus recommended that future studies investigate the impact of exposure on mechanistic pathways related to pesticides and diseases.

5. Conclusion

The study identified a profile of circulating miRNAs that are associated with pesticide exposure. The study revealed that the negative regulation of miR-423-3p, miR-1193, miR-576-5p, miR-509-5p, miR-548b-3p, miR-1469, miR-329-3p, and miR-548j-3p, and the positive regulation of miR-301b-5p and miR-548ah-5p in patients with CLL and MM were associated with occupational exposure to pesticides. The integrated network analysis between the differentially expressed miRNAs in the group of patients with exposure and their target genes demonstrated that miRNAs can participate in the regulation of signaling pathways, such as PI3K/Akt/mTOR and MAPK. Additionally, these miRNAs were found to be associated with an increased risk of developing

neurological diseases and various types of cancer. The expression of miRNAs was primarily influenced by the exposure factor in the multivariate regression analysis. Our findings provide new information on epigenetic changes related to pesticide exposure, which may contribute to the development of preventive environmental monitoring and diagnosis. The plasma miRNAs identified may serve as promising candidates for biomarkers of occupational exposure to pesticides, potentially contributing to the emerging field of Precision Environmental Health.

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Author Contributions

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Data Availability Statement:

Due to the sensitive nature of the questions asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

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4.3 Artigo 3- Occupational exposure to pesticides reveals a miRNAs expression profile indicating multiple disease risks

Occupational exposure to pesticides reveals a miRNAs expression profile indicating multiple disease risks

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Abstract

A growing body of evidence indicates a correlation between exposure to pesticides and adverse effects on human health. Such exposure has been linked to the development of pulmonary, cardiovascular, and neurodegenerative diseases, as well as various forms of cancer. The objective of this study was to evaluate the miRNA expression profile in individuals exposed to pesticides and to predict the biological functions of differentially expressed miRNAs through target gene analysis. The quantification of pesticides in the serum of the individuals revealed a significant difference in the concentration of dichlorodiphenyldichloroethylene, between the two groups. Cellular analysis revealed a higher prevalence of micronucleated, binucleated, pyknotic, and karyolytic cells in the exposed group. Furthermore, the plasma miRNA expression profile yielded 30 miRNAs that exhibited differential expression between the exposed and non-exposed groups. Following ROC curve analysis (AUC $\geq$ 0.75), 20 miRNAs with significant differential expression ($p < 0.005$) were selected for subsequent functional analyses. A total of 1,004 validated target genes were predicted for the miRNAs. The analysis yielded 213 biological process terms, 131 cellular component terms, and 170 molecular function terms. A KEGG pathway enrichment analysis identified 91 enriched pathways for the predicted target genes. Moreover, an examination of the correlation between the target genes and disease revealed the presence of 107 diseases, with the most prevalent being neurological disorders, arthritis, and cancer. Our findings provide insights into the biological pathways affected by pesticide and underscore the critical role of miRNAs in the pathogenesis of related diseases, particularly neurological conditions and cancers.

Keywords: Pesticides, miRNAs, occupational exposure, genomic instability.

1. Introduction

Pesticides are defined as a substance or mixture of substances used extensively in agricultural production globally with the primary objective of eradicating diverse living organisms perceived as detrimental to agricultural productivity (Bitencourt de Morais Valentim et al., 2023; Damalas and Koutroubas, 2016). Brazil is among the world's leading consumers of pesticides. Such exposure may occur in both an environmental and occupational context (Gama et al., 2022). Pesticide contamination may occur via three principal routes: contact, ingestion, and inhalation. Those engaged in agricultural activities who have direct contact with pesticides are particularly susceptible to the adverse effects associated with these compounds. Conversely, exposure to these substances is also prevalent among the general population, primarily through the ingestion of contaminated water and food (Bitencourt de Morais Valentim et al., 2023; MacFarlane et al., 2013).

The existing literature indicates a correlation between exposure to pesticides and an increased risk of developing neurodegenerative disorders and cancer (Dardiotis et al., 2019; Gama et al., 2022; Torres-Sánchez et al., 2023). As documented in the literature, exposure to pesticides is regarded as a risk factor for a number of different types of cancer (Alavanja et al., 2014; Andreotti et al., 2020; Francisco et al., 2023; Lerro et al., 2021; Martin et al., 2018; Pardo et al., 2020; Tual et al., 2019; Ventura et al., 2019). The International Agency for Research on Cancer (IARC) has published findings indicating the potential role of certain pesticides in carcinogenesis, particularly those classified as persistent organic pollutants (POPs), such as DDT and lindane. Furthermore, several currently utilized pesticides, including 2,4-D, diazinon, glyphosate, and malathion, have been identified as potentially carcinogenic, indicating a potential threat to the exposed population (IARC, 2018, 2015). Nevertheless, despite epidemiological studies indicating an association between pesticide exposure and the development of diseases such as cancer, the biological mechanism underlying this link remains unclear.

Genomic instability has been demonstrated to play a pivotal role in the pathogenesis of cancer and other diseases (Hanahan, 2022). A number of studies have assessed the impact of occupational exposure to pesticides on genomic stability, with the objective of establishing the genetic effects of such exposure (Andreotti et al., 2015; Benedetti et al., 2013; Cobanoglu et al., 2019; dos Santos et al., 2022; Kahl et al., 2018). This may provide evidence indicating a correlation between such exposure and the development of a variety of diseases, including cancer. Accordingly, among the

biomarkers of genomic instability, the micronucleus test is particularly suited to measuring the cytotoxic effects of occupational and environmental exposure to pesticides. In this context, studies conducted in Brazil have demonstrated that populations occupationally exposed to pesticides exhibit a higher prevalence of micronuclei, as well as an elevated frequency of cell death and chromosomal damage, in comparison to individuals not exposed to pesticides (Benedetti et al., 2013; Da Silva et al., 2012; dos Santos et al., 2022; Kahl et al., 2018; Marcelino et al., 2019; Silveira et al., 2013).

Furthermore, given the role of epigenetic effects in the development of cancer and other diseases, classes of epigenetic markers, such as miRNAs, have been identified as potential biomarkers of occupational exposure in recent years. miRNAs are a class of short non-coding RNA approximately 22 nucleotides in length that play an active role in epigenetic regulation of gene expression and are also involved in post-transcriptional gene silencing (Marsit et al., 2006; Vrijens et al., 2015). Additionally, miRNAs are associated with a multitude of biological and molecular processes, including cell proliferation, differentiation, intracellular communication, and apoptosis (Collotta et al., 2013; Nolte-'t Hoen et al., 2015). Moreover, they can be detected in a range of biological fluids (Weber et al., 2010).

A limited number of studies have evaluated the relationship between pesticide exposure and miRNA expression in humans (Gattuso et al., 2022; Krauskopf et al., 2017; Weldon et al., 2016). In their study, (Gattuso et al., 2022), valuated the expression of hsa-miR-199a-5p in agricultural workers exposed to pesticides. In contrast, Weldon et al. (2016) evaluated the presence of miRNAs in the urine of 27 parent/child, agricultural worker/non-agricultural worker pairs. The study by Krauskopf et al. (2017) evaluated the miRNA response observed in a human population exposed to POPs, including the pesticides hexachlorobenzene (HCB) and dichlorodiphenyltrichloroethane (DDT). Nevertheless, previous research has identified alterations in miRNAs in response to specific types of chemical exposure and environmental contamination. These include particulate matter (Bollati et al., 2010), metals and PAHs (Deng et al., 2019), organic solvents (Sisto et al., 2019), tobacco use (Badrnya et al., 2014; Takahashi et al., 2013), trichloroethylene (Lee et al., 2019), asbestos (Jia et al., 2019) and vinyl chloride monomer (Feng et al., 2017). In light of these findings, further research employing miRNAs can enhance our comprehension of the underlying molecular mechanisms of pesticide exposure and its associated risks to human health.

To this end, we conducted a cross-sectional study with the objective of identifying the plasma miRNA expression profiles in pesticide applicators in order to detect potential biomarkers of exposure. By predicting the target genes of miRNAs, the potential biological and functional roles of miRNAs became apparent through the analysis of various databases. Furthermore, the study investigated the genotoxic and cytotoxic effects of pesticide exposure on individuals occupationally exposed to these substances.

2. Material and methods

2.1 Study population

Participants for the study were selected from a cohort included in the Rural Workers and Cancer: a Cohort Study (RUCAN). The RUCAN cohort is a prospective Brazilian study that is currently being conducted in the Barretos region of the state of São Paulo, Brazil. The objective is to recruit individuals with and without exposure to pesticides. Moreover, this cohort is part of the International Consortium of Agricultural Cohorts (AGRICOH) (https://agricoh.iarc.fr/participating-cohorts/). Consequently, a total of 46 individuals were selected for inclusion in the study, comprising 23 rural workers who apply pesticides and 23 non-occupationally exposed individuals from the Barretos region in Brazil. Given that the majority of pesticide applicators in the region were male, only male participants aged between 18 and 75 were included in the study. A standardized questionnaire, adapted from the Agricultural Health Study (AHS) (https://aghealth.nih.gov/), was utilized to collect data pertaining to demographics, health status, lifestyle, and occupational exposure. The study population consisted of individuals with no history of cancer and who had resided in the same study area for a minimum of one year. The exposed group consisted of individuals who had been exposed to pesticides for a minimum of two years and reported no history of exposure to other carcinogenic compounds utilized for non-agricultural purposes. Furthermore, the non-exposed group consisted of participants who were matched by age, history of smoking and alcoholism, in addition to having no history of contact with pesticides or other agents deemed carcinogenic. All data were stored on the RedCap platform. Peripheral blood and oral mucosa samples were concurrently collected with the administration of the questionnaire and subsequently transported to the laboratory for storage until subsequent analysis. Subsequently, the participants were instructed to rinse their mouths with water, after which oral exfoliated cells were collected from both groups using a cytobrush (BD

TriPath Imaging, Burlington, N.C., USA). The brush heads were placed directly into vials containing an alcohol-based solution (SurePath, BD TriPath Imaging) and transported to the laboratory, held at room temperature in accordance with the methodology described by Silva et al. (2019). Venipuncture was employed to collect the blood samples, which were then transported at a temperature of 8°C or lower to the Biobank of the Barretos Cancer Hospital for processing and storage (Neuber et al., 2022). The study was approved by the Ethics Committee of the Barretos Cancer Hospital (protocol number 00270418.2.0000.5437).

2.2 Determination of pesticide exposure in serum

Analyses were performed by Gas Chromatography-Mass Spectrometry Tandem with an Agilent 7890 Gas Chromatograph coupled to a Waters Quattro Micro GC mass spectrometer employing a SLB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 μm). Aliquots of 400 μL of serum, thawed at room temperature, were prepared with the addition of organic solvents, an internal standard, and sonication. The extraction was performed using Solid Phase Extraction with a Waters Oasis Prime HLB 3cc (60 mg) cartidge. Derivatization was conducted using MTBSTFA and 1% TBDMSCl. The 23 identified compounds are presented in Table S1. The linearity, as indicated by the R2 value, exceeded 0.982, with the LOQ spanning a range of 0.3 to 5.6 ng/mL^{-1}. In instances where values fell below the LOD, they were replaced with the LOD divided by the square root of two, as proposed by (Hornung and Reed, 1990). The intraday accuracy was found to be within the range of 77-123%, while the interday values were observed to be within the range of 69-124%. The precision of the method was determined to be an intraday coefficient of variation of 2 to 28% and an interday coefficient of variation of 4 to 21%. This method has been previously described (Birolli et al., 2024).

2.3 Buccal micronucleus cytome assay

The buccal micronucleus (MNC) cytome assay was described according to Silveira et al. (2013). Buccal mucosa samples were obtained from each subject using a cytobrush (BD TriPath Imaging, Burlington, NC, USA) agitated in Surepath preservative liquid (BD TriPath Imaging, Burlington, NC, USA). Subsequently, the cells were resuspended and transferred to the slides for further analysis. The slides were then subjected to a drying process prior to the application of Schiff's reagent for staining and Fast Green for counterstaining. Two slides were prepared for each individual, with a

coverslip mounted using Entellan. The number of MNC was counted for 2000 cells, while 1000 cells were counted to determine the frequency of binucleated cells, karyolysis, pyknotic nuclei, and nuclear budding (Thomas et al., 2008). The normality of the MNC data was evaluated through the application of the Shapiro-Wilk test. Thereafter, the Wilcoxon test was employed to assess the discrepancy in the parameters assessed in the MNC test between the exposed and non-exposed groups. All analyses were conducted with a statistical significance threshold of $p < 0.05$.

2.4 miRNA expression profile

In this study, we conducted an analysis of circulating miRNAs in plasma from a blood sample collected in an EDTA tube from each study participant. The miRNA extraction was performed on 400 µL of plasma using the miRNeasy serum/plasma miRNA isolation kit. The analysis of miRNA expression was performed using the nCounter® Human v3 miRNA assay (NanoString Technologies, Seattle, USA). Approximately 100 ng of total RNA from each sample was subjected to tag binding and hybridization with the assay's Reporter CodeSet and Capture ProbeSet. Following hybridization, the samples were processed using the NanoString PrepStation and immobilized onto the nCounter cartridge. The cartridge was then loaded into the nCounter® Digital Analyzer, where image capture and data acquisition were conducted.

2.5 Data normalization and bioinformatics analysis

Data collection was conducted on the nCounter Digital Analyzer (NanoString Technologies, Seattle, WA, USA) in accordance with the manufacturer's instructions. The raw miRNA values were normalized using the miRNA counting method with the lowest coefficient of variation (low CV) via the NanoStringNorm package in the R statistical environment (R-project, Vienna, Austria). For differential expression analysis, the "limma" package from Bioconductor in the R environment (Ritchie et al., 2015) was utilized. The expression profile of the miRNAs was depicted in heatmaps using the ComplexHeatmap package (Gu et al., 2016), and the fold change (FC) value > 1 or < -1 was employed to ascertain the positive or negative regulation of the differentially expressed miRNAs.

2.6 Target prediction and functional analysis of miRNAs target genes

For all relevant miRNAs associated with exposure in our study, we conducted a search for validated target genes using the mirDIP database (https://ophid.utoronto.ca/mirDIP/). In silico putative target prediction of the selected miRNAs was conducted using the following databases: DIANA, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and Targetscan. The target genes present in at least five of the aforementioned databases were selected for further enrichment analysis. The Gene Ontology, KEGG, and DisGeNET enrichment analyses were conducted using the DAVID 6.8 Beta Knowledgebase database (https://david-d.ncifcrf.gov/). The relevance of the enrichment of the set of genes analyzed was assessed using a p-value of <0.05, which indicated a significant over-representation. To facilitate the visual representation of the miRNA-mRNA interaction, an interaction network was constructed using the Cytoscape v3.9.1 software.

2.7 Statistical Analysis

The analyses were conducted using R version 4.4.4 on the R Studio platform (R Core Team, 2020). Qualitative variables were characterized using frequency and proportion, while quantitative variables were characterized using mean and standard deviation.

In order to compare qualitative variables, the Chi-square test (parametric) and Fisher's exact test (non-parametric) were employed. In the case of quantitative variables, comparisons between two groups were conducted using the T-test (parametric) and the Wilcoxon test (non-parametric). For comparisons between more than two groups, both Analysis of Variance (ANOVA) and the Kruskal-Wallis Test (non-parametric) were employed. To evaluate the correlation between the quantitative variables associated with the exposed group, a Spearman's correlation and a probability value were calculated. Moreover, to determine the functional relationship between the numerical response variables and the covariates, a multiple linear regression analysis was conducted, with the parameter estimates presented in forest plot graphs, including their respective confidence and significance intervals.

The relationship between the miRNAs was interpreted through the use of a Principal Components Analysis (PCA), which generated biplots (PCA 1 x PCA 2) for both individuals and variables. Furthermore, a graph was constructed to demonstrate the eigenvectors of the original variables and their respective contributions to the first two

principal components. Additionally, a ROC analysis was conducted on all the miRNAs to evaluate potential threshold values for classifying them in relation to the groups. This was accomplished by employing the measures of sensitivity, specificity, and AUC for the candidates under consideration for miRNA classification values. In all statistical tests, a significance level of 0.05 (five percent) probability was adopted.

3. Results

3.1 Characteristics of the study population

Table 1 presents a summary of the demographic characteristics of the 46 participants included in the study. Of the total number of participants, 23 had been exposed to pesticides, while the remaining 23 had not. The majority of participants identified as white, reported alcohol consumption, with no reports of tobacco use. The mean age of the participants was 51 years. No statistically significant differences were observed between the two groups with regard to ethnicity, age, tobacco use, or alcohol consumption. The mean duration of exposure to pesticides among those exposed was 33 years (Table 1).

Table 1. Socio-demographic characteristics of individuals exposed and non-exposed to pesticides.

Characteristics	**Exposed** (n = 23)	**Non-exposed** (n = 23)	**p-value**[1]
Ethnicity			
White	15 (65%)	14 (61%)	
Black	3 (13%)	4 (17%)	>0.9
Brown	5 (22%)	5 (22%)	
Age (mean ± SD)	51 ± 11	51 ± 12	0.8
Smoking status			
Current	5 (22%)	5 (22%)	
In past	7 (30%)	5 (22%)	0.8
Never	11 (48%)	13 (57%)	
Alcohol consumption			
No	5 (22%)	5 (22%)	>0.9
Yes	18 (78%)	18 (78%)	
Time of exposure to pesticides in years (mean ± SD)	33 ± 14	-	

Note: SD: standard deviation.
[1]Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test.

3.2 Quantification of pesticides in serum

The concentrations of 22 active ingredients were quantified in the serum of occupationally exposed and non-exposed individuals (Table S1). A statistically significant

difference was observed between the two groups with regard to the DDE compound (p = 0.007), with the exposed group exhibiting a higher average concentration (Table 2). It is noteworthy that the majority of the compounds quantified belong to the organochlorine class (Table S1).

Table 2. Quantification of the pesticide metabolites examined in serum samples from the participants.

Pesticides	**Non-exposed** (n = 23) (Mean ± SD)	**Exposed** (n = 23) (Mean ± SD)	**p-value**[1]
Dichlorodiphenyldichloroethylene (DDE)	1.05 ± 0.52	2.49 ± 2.81	**0.007**
Alpha Endosulfan	1.06 ± 0.39	1.27 ± 0.50	0.2
BetaEndosulfan	3.14 ± 1.12	3.63 ± 1.99	0.6
Methoxychlor	0.83 ± 1.62	0.17 ± 0.81	0.091
Lindane and Beta HCH	2.13 ± 1.64	3.15 ± 2.58	0.2
4-Nitrophenol (PNP)	0.95 ± 0.27	1.00 ± 0.31	0.6
3-Phenoxybenzoic acid (3- PBA)	0.77 ± 0.10	0.88 ± 0.25	0.2
2.4- Dichlorophenol	0.6 ± 2.8	2.8 ± 6.4	0.2
PhenylPyrazol	0.32 ± 0.42	0.47 ± 1.05	0.4
Fipronil sulfone (FipSul)	1.7 ± 2.9	2.2 ± 4.7	0.7
3.5.6-Trichloro-2-pyridinol (TCP)	0.77 ± 0.64	1.17 ± 1.13	0.2

[1]Wilcoxon rank sum test.

3.3 Biomarkers of genomic instability, cytokinesis failure and cell death

The Buccal micronucleus cytome assay demonstrated that the individuals in the pesticide-exposed group exhibited a higher prevalence of cells with MNC, binucleated, pycnotic, and in karyolysis when compared to the non-exposed group (Figure 1). No significant difference was observed in the nuclear budding parameter between the two groups. These findings demonstrate that the cells from individuals exposed to the pesticide exhibit elevated levels of genotoxicity, cytokinesis failure, and cell death in comparison to the cells from individuals who were not exposed.

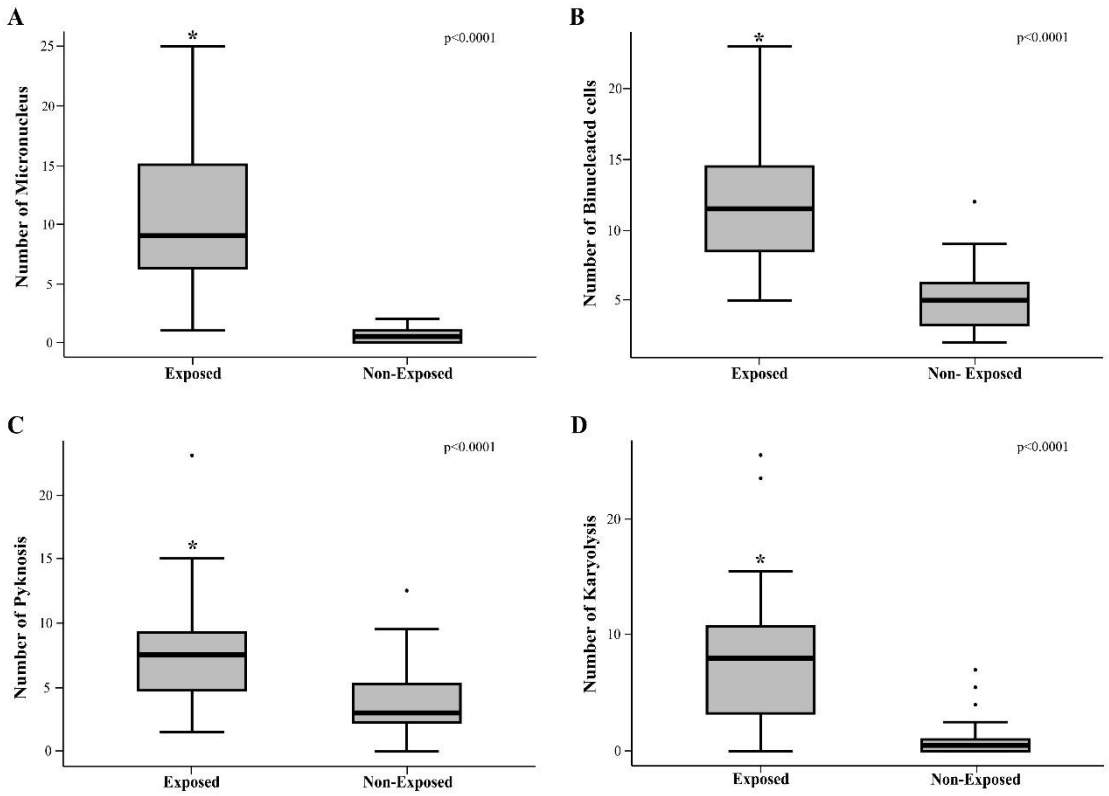


Figure 1. Analysis of the number of cells with A) micronucleus, B) binucleated, C) in pyknosis, D) in caryolysis, in the group of exposed individuals compared to the group of individuals not exposed to pesticides.* p<0.0001 per Wilcoxon test.

3.4 Correlation between biomarkers of genomic instability, cytokinesis failure, cell death, and predictive variables

The Spearman correlation coefficients for genotoxicity, cytokinesis failure, cell death biomarkers, and quantitative dependent variables in the pesticide-exposed group are shown in Figure S1. The analysis revealed a positive correlation between MNC and age (r = 0.45, p = 0.03). Binucleated cells exhibited a positive correlation with age (r = 0.60, p = 0.002) and exposure time (r = 0.52, p = 0.01), yet displayed a negative correlation with the metabolite PhenylPyrazol (r = -0.41, p = 0.04). A negative correlation was observed between nuclear buds and exposure duration (r = -0.46, p = 0.02) and the metabolite Alpha-Endosulfan (r = -0.46, p = 0.02). Moreover, pyknosis exhibited a positive correlation with the metabolite Endrin (r = 0.44, p = 0.03). Among the biomarkers assessed, only karyolysis demonstrated no significant correlation with the analyzed variables (Figure S1). The correlation analysis of all cytome assay parameters revealed a significant positive correlation between binucleated cells and MNC (r = 0.41, p = 0.04) and a negative correlation between binucleated cells and nuclear buds (r = -0.55, p = 0.006) (Figure S2). Significant correlations ($p \leq 0.05$) are shown in Figure 2.

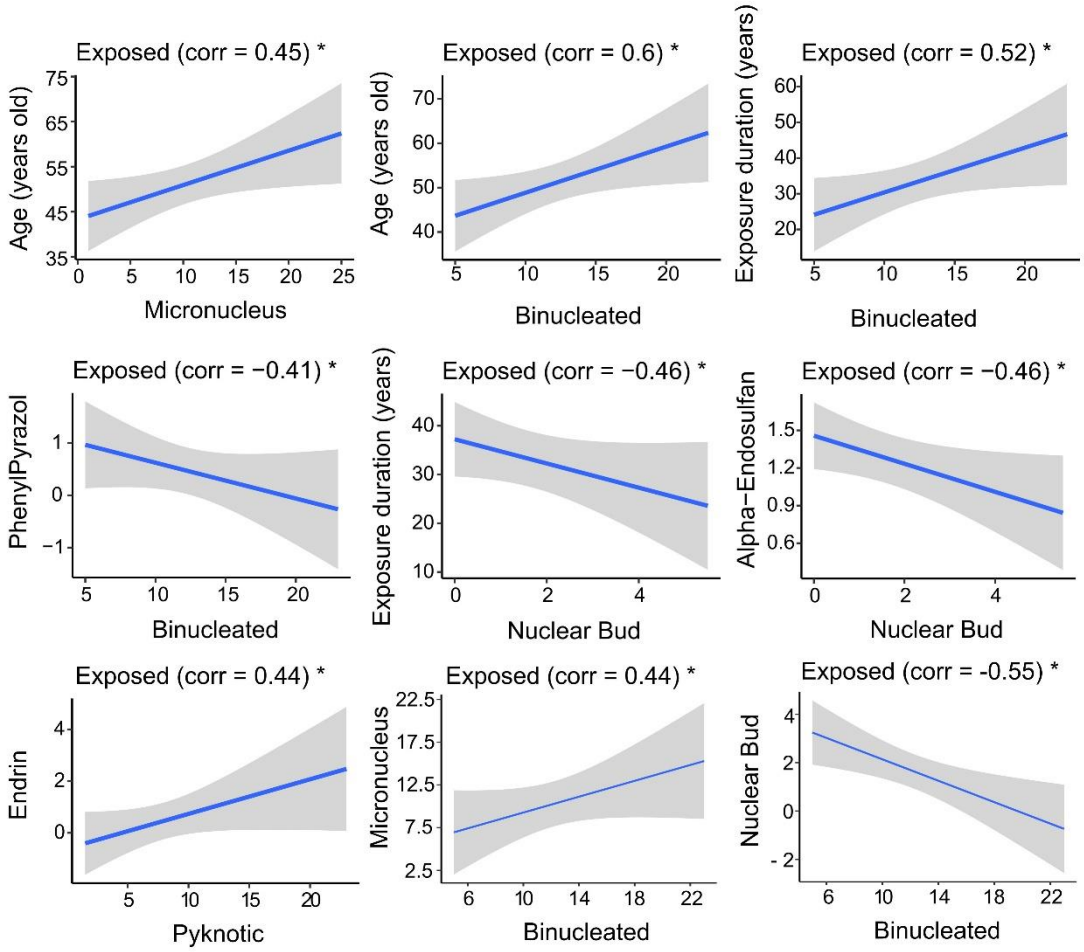


Figure 2. Significant Spearman correlation coefficients between the markers of genotoxicity, cytokinesis failure and cell death, and the quantitative dependent variables in the pesticide exposed workers group. * p< 0.05.

Upon analysis of the qualitative variables, including ethnicity, tobacco use, alcohol consumption, pesticide intoxication, and duration of exposure, no significant influence was observed on the count of parameters assessed by the micronucleus test ($p > 0.05$) (Figure S3).

3.5 Multiple linear regression analysis of biomarkers of genomic instability, cytokinesis failure, and cell death

A multiple linear regression analysis was subsequently conducted to determine the influence of potential factors on the parameters of genomic instability, cytokinesis failure, and cell death in the pesticide-exposed group (Figure 3, Table S2). The most significant variables for the final models of each parameter were exposure group for MNC ($\beta = 0.739$ and $p = <0.0001$) and nuclear budding ($\beta = 0.765$ and $p = <0.0001$), binucleated cells for pyknosis ($\beta = 0.534$ and $p = <0.0001$) and karyolysis ($\beta = 0.799$ and $p = <0.0001$).

Additionally, MNC and karyolysis for binucleated cells (β = 0.368 and p = <0.0001, and β = 0.453 and p = <0.0001, respectively) were identified as significant variables (Figure 3, Table S2).

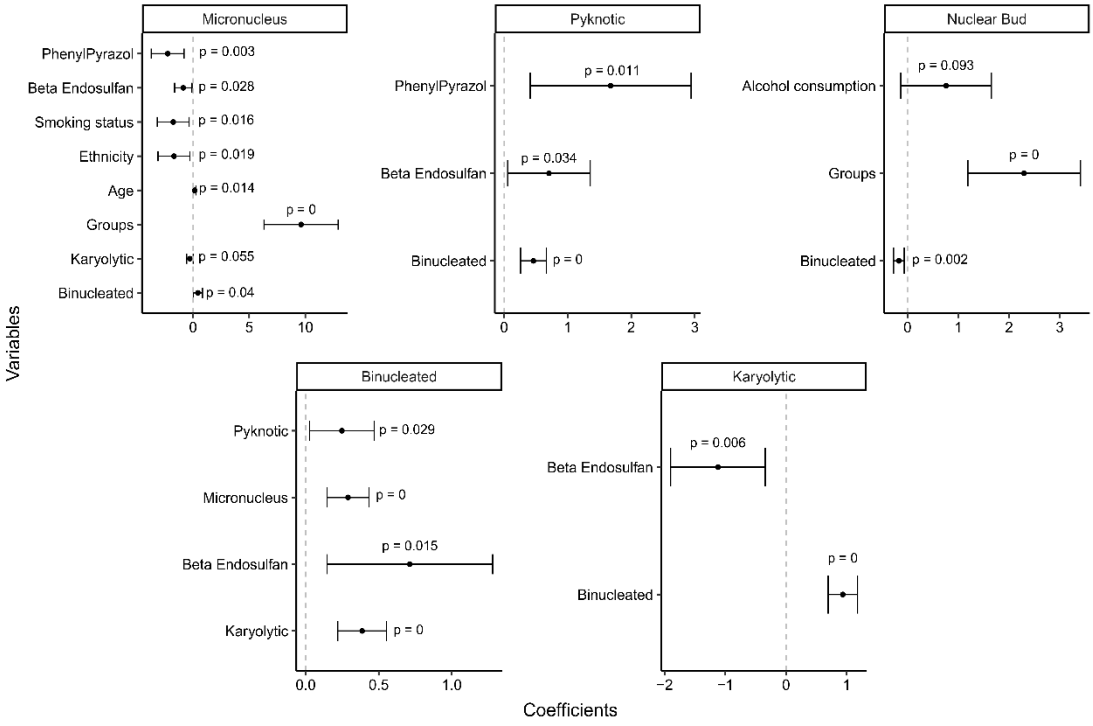


Figure 3. Forest plot of multiple linear regression analysis illustrates the potential variables that may significantly contribute to the observed increase in micronucleus, nuclear budding, binucleate cells, pyknosis, and karyolytic changes.

3.6 Expression profile of miRNAs

The analysis of differentially expressed miRNAs between the pesticide-exposed and non-exposed groups revealed that a total of 109 miRNAs exhibited differential expression between the two groups ($p \leq 0.05$), comprising 13 upregulated and 96 downregulated miRNAs (Table Excel S1). To identify miRNAs for further investigation of their biological functions, a p-value threshold of $p \leq 0.005$ was applied to narrow down the selection. This process identified 30 differentially expressed miRNAs, of which 29 were downregulated and 1 was upregulated (Figure 4).

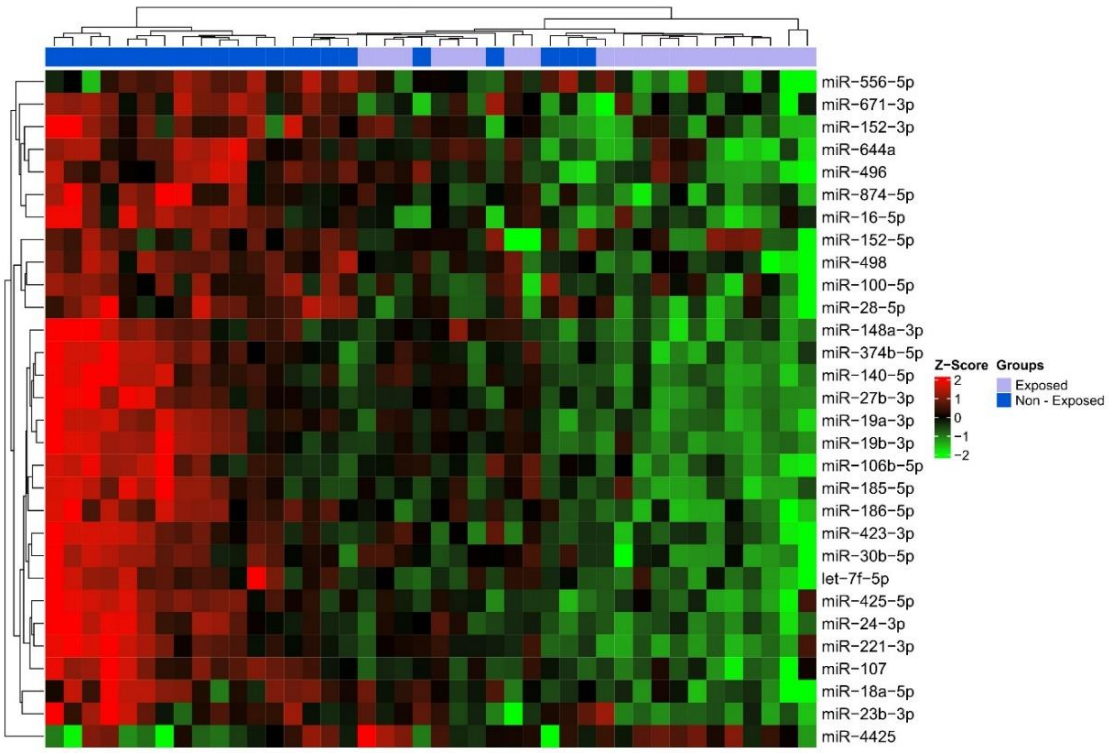


Figure 4. Heatmap of 30 differentially expressed miRNAs, generated using unsupervised hierarchical clustering. The discrepancy in miRNA expression between the two groups was assessed using the t-test ($p < 0.005$). The coloration of the image represents the degree of expression: red denotes up-regulated miRNAs, while green represents down-regulated miRNAs.

3.7 Differentially expressed miRNAs as potential biomarkers of pesticide exposure

A receiver operating characteristic (ROC) curve analysis was conducted on the thirty differentially expressed microRNAs to evaluate their potential for discriminating between the exposed and non-exposed groups. Of the 30 miRNAs, 20 demonstrated an area under the curve (AUC) of ≥0.75, indicative of robust discriminatory capacity (Figure 5, Table S3). The 20 miRNAs were consistently observed to be downregulated in the exposed group relative to the non-exposed group (hsa-miR-556-5p, hsa-miR-100-5p, hsa-miR-28-5p, hsa-miR-874-5p, hsa-miR-18a-5p, hsa-miR-498, hsa-miR-374b-5p, hsa-miR-221-3p, hsa-miR-27b-3p, hsa-miR-24-3p, hsa-miR-425-5p, hsa-miR-107, hsa-miR-148a-3p, hsa-miR-186-5p, hsa-miR-19a-3p, hsa-miR-106b-5p, hsa-let-7f-5p, hsa-miR-423-3p, hsa-miR-30b-5p, and hsa-miR-23b-3p) (Table S3). These findings suggest that the 20 miRNAs in question are effective at distinguishing the pesticide-exposed group from the non-exposed group based on their expression profiles.

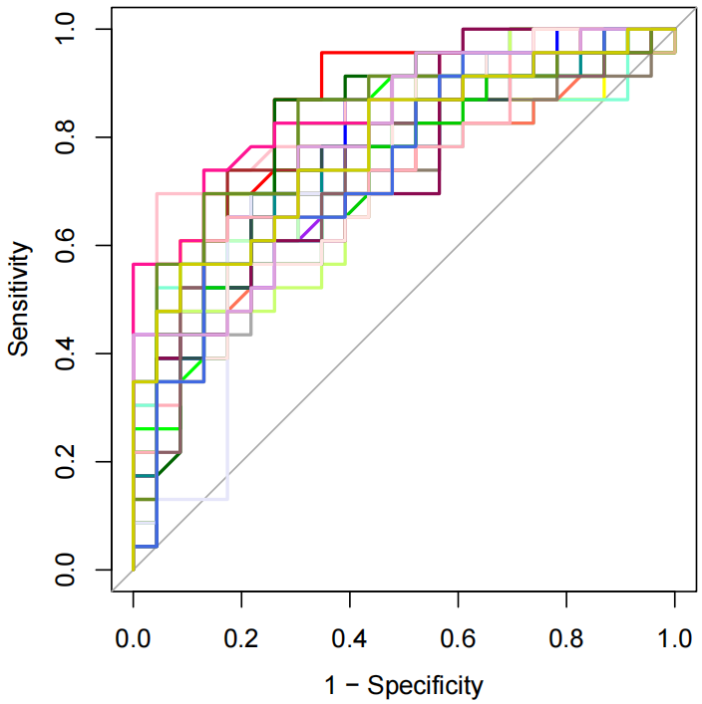


Figure 5 - Receiver operating characteristic (ROC) curve of the thirty miRNAs differentially expressed between the pesticide-exposed and non-exposed groups for predicting potential biomarkers. Each line represents the AUC value of each miRNA evaluated.

3.8 Correlation of differentially expressed miRNAs in the exposed group and predictor variables

Correlation analyses were conducted on the 20 miRNAs with a p-value < 0.005 and AUC ≥ 0.75. The pesticide metabolites quantified in serum were selected as the relevant exposures of interest. The following pesticides were included in the analysis: DDE, Dieldrin, Alpha-Endosulfan, Beta-Endosulfan, DDD, DDT, Methoxychlor, Endrin, Gamma and Beta-HCH, 4-Nitrophenol, 3-Phenoxybenzoic acid, 2,4-Dichlorophenol, PhenylPyrazol, FipSul, and TCP. The Spearman correlation coefficients between these metabolites and plasma miRNA levels in individuals exposed to pesticides are presented in Figure S4. Correlations were generally < 0.7. Significant correlations ($p < 0.05$) are shown in Figure 6. The highest significant coefficients were observed for TCP with miR-30b-5p ($r = -0.64$, $p = 0.001$) and miR-874-5p ($r = -0.67$, $p = 0.0005$), and for PhenylPyrazol with miR-374b-5p ($r = 0.62$, $p = 0.001$) and miR-221-3p ($r = 0.64$, $p = 0.001$) (Figure S4).

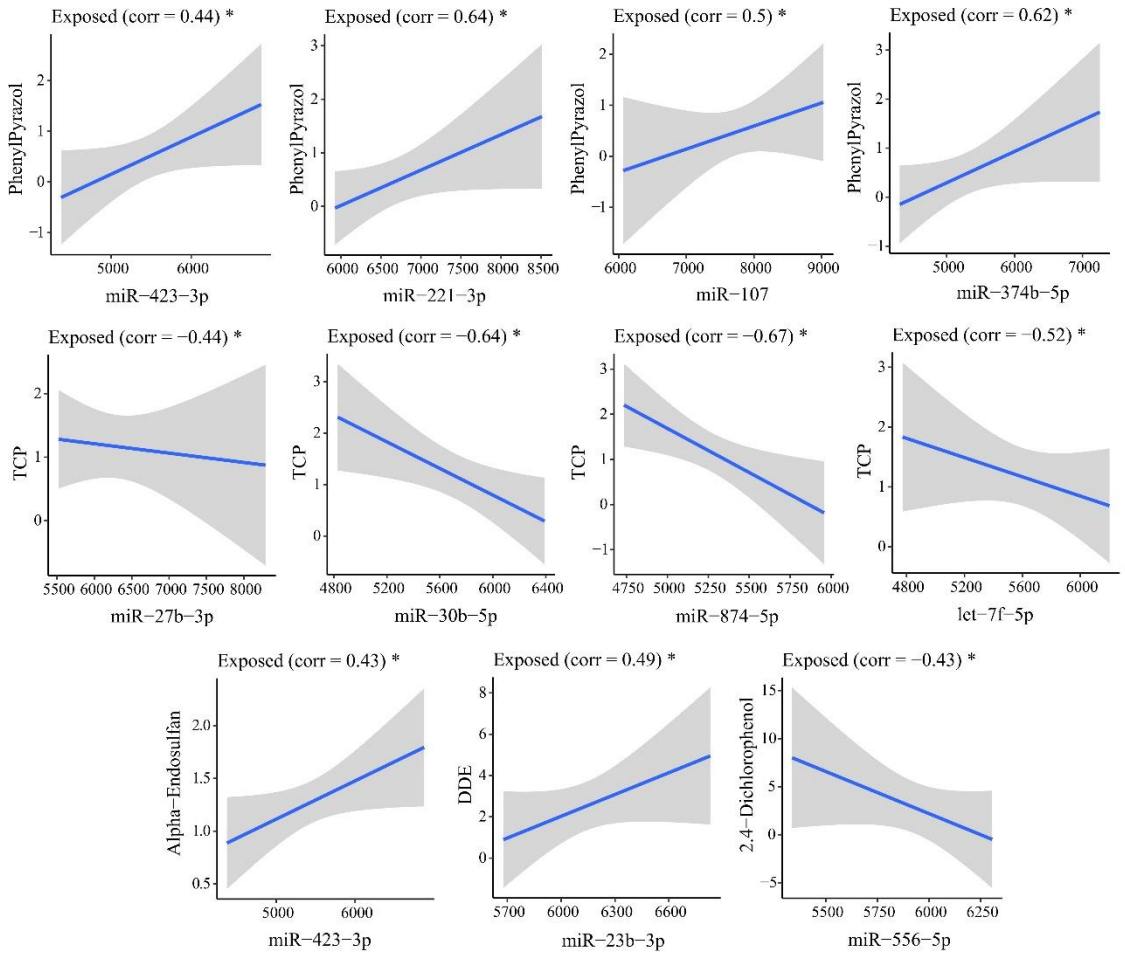


Figure 6. Significant Spearman correlation coefficients between the expression levels of miRNAs and the concentrations of pesticides quantified in the serum of workers exposed to pesticides. * p< 0.05.

Figure S5 illustrates the correlation coefficients between miRNA levels and a number of parameters, including genomic instability, cytokinesis failure, cell death, age, and exposure duration, in the pesticide-exposed group. Significant negative correlations were observed between miR-28-5p expression and nuclear budding (r = -0.43, p = 0.04), miR-423-3p and karyolysis (r = -0.44, p = 0.03), and miR-186-5p and MNC (r = -0.43, p = 0.03). A significant positive correlation was identified between miR-498 and exposure duration (r = 0.46, p = 0.02) (Figure S5). The analysis of miRNA expression differences within qualitative dependent variables in the pesticide-exposed group revealed significant differences associated with ethnicity, tobacco use, alcohol consumption, and pesticide intoxication events (Figure 7).

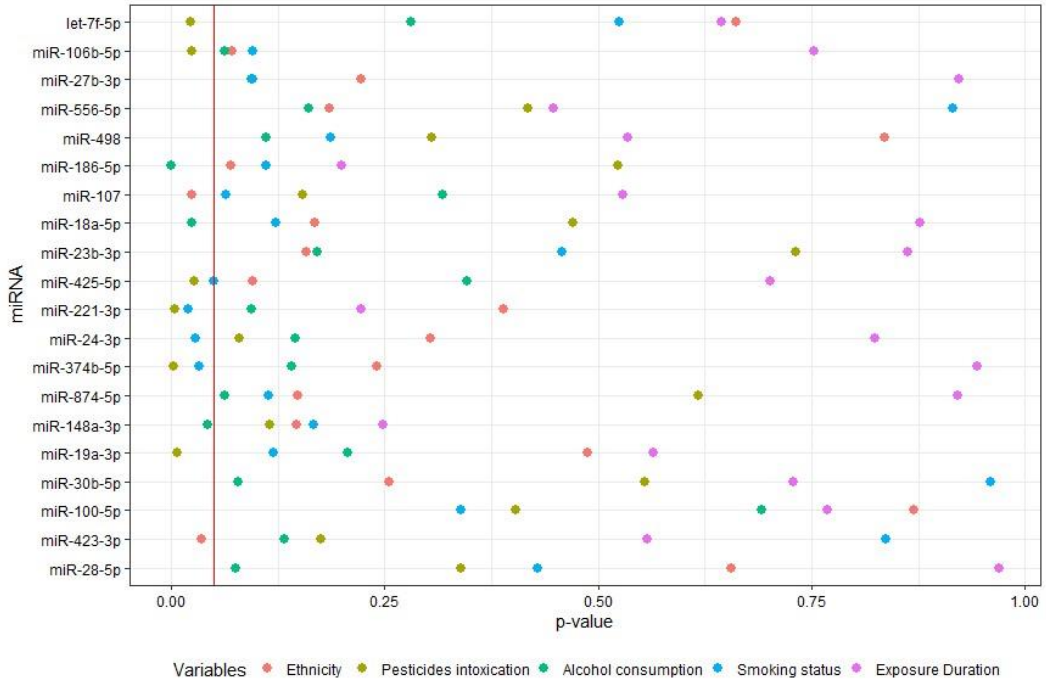


Figure 7. Dot plots for each miRNA and their respective probability values from the statistical tests (horizontal axis) for the qualitative variables of ethnicity, pesticide toxicity, alcohol consumption, tobacco use, and exposure duration in the group of pesticide-exposed workers. The T-test, Wilcoxon, ANOVA, and Kruskal-Wallis tests were used for each distribution assumption and number of levels for the variables. The vertical line in red indicates the significance level used ($p<0.05$).

A comparative subgroup analysis of individuals exposed to pesticides revealed significant differences in miRNA expression. Individuals of self-reporting black ethnicity exhibited lower expression levels of miR-423-3p and miR-107. The expression of miR-148a-3p, miR-18a-5p, and miR-186-5p was found to be reduced in individuals who consume alcohol, while current tobacco users exhibited reduced expression of miR-374b-5p, miR-24-3p, miR-221-3p, and miR-425-5p. In contrast, individuals who reported incidents of pesticide exposure exhibited elevated expression levels of miR-19a-3p, miR-374b-5p, miR-221-3p, miR-425-5p, miR-106b-5p, and let-7f-5p relative to those without such reports (Figure 8).

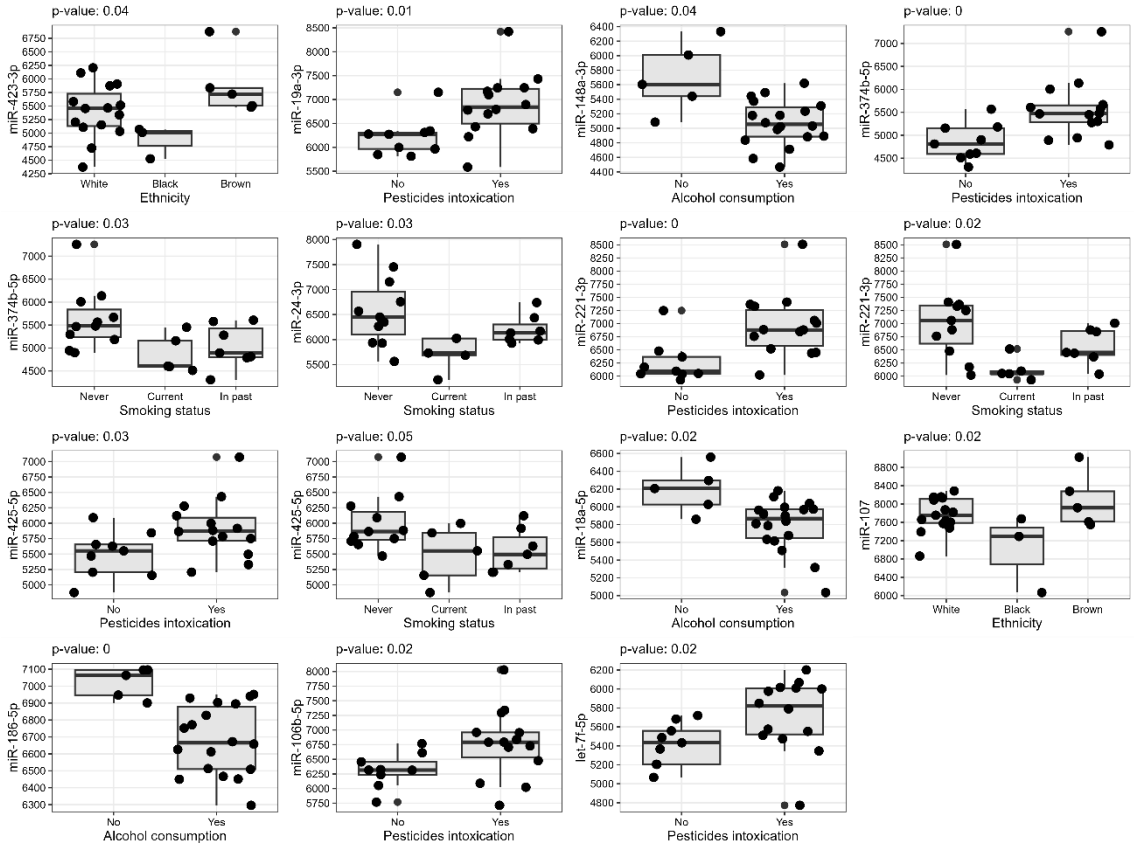


Figure 8. Comparison of mean miRNA expression levels of workers exposed to pesticides for each variable shown to influence expression.

3.9 PCA analysis between groups of individuals and biomarkers

Figure 9 depicts the PCA results, which illustrate a spatial distribution of miRNA expressions and cytome assay parameters for the purpose of evaluating the relative implication of these data in each sample. The plot of scores illustrates the position of each sample in the ordination plane of the two significant principal components (F1 and F2), which respectively account for 15.2% and 11.1% of the total variation (Figure 9A). The PCA also indicated a segregation between the exposed and non-exposed groups, predominantly driven by the primary component F1 (Figure 9A). F1 was primarily loaded by cytome assay parameters (binucleated, MNC, karyolitics, and pyknotic), while exhibiting a negative loading for miR-374b-5p, let-7f-5p, miR-19a-3p, and miR-24b-3p. While component F2 was primarily loaded by miR-27b-3p, miR-106b-5p, pyknotic, and binucleated, and negatively loaded by let-7f-5p and miR-425-5p (Figure 9B and Figure S6).

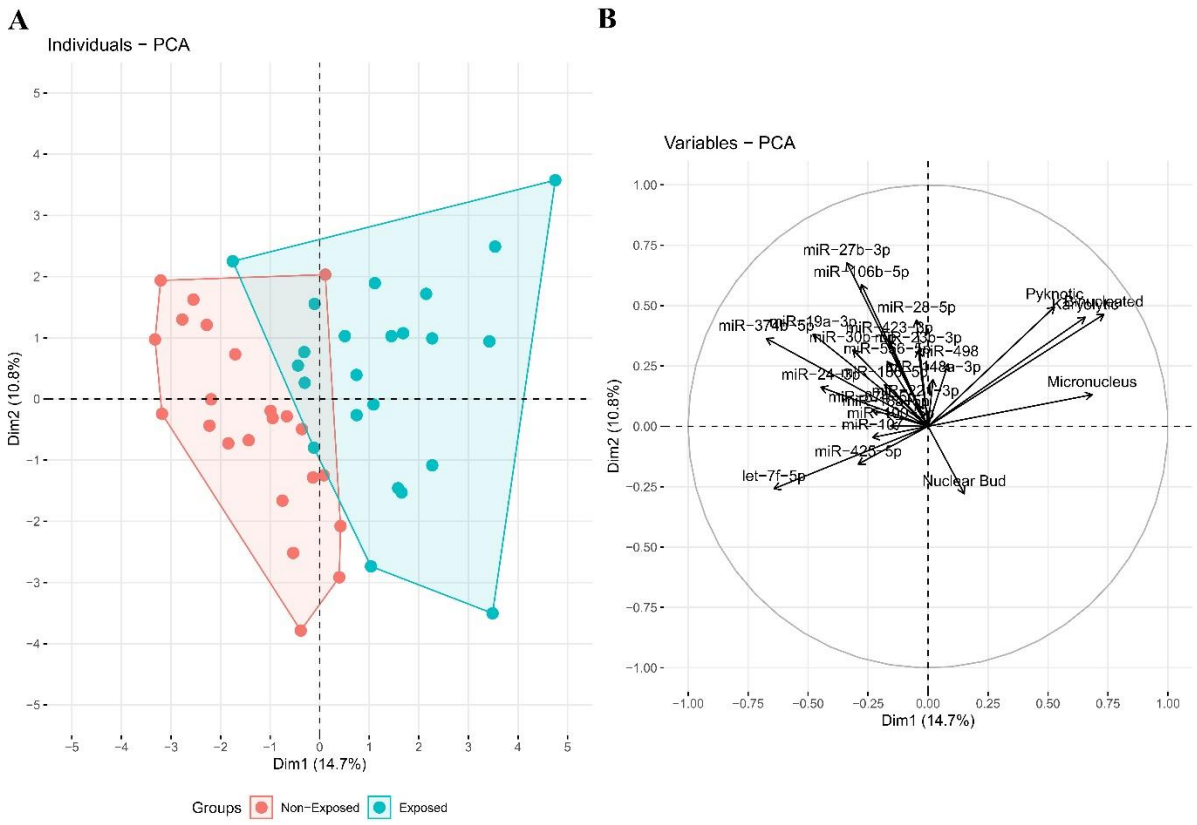
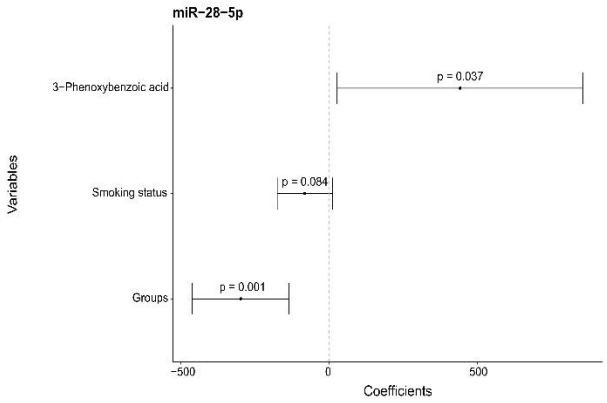


Figure 9. Principal component analysis (PCA) of plasma miRNAs between the pesticide-exposed and non-exposed groups. (A) Clustering between the samples of the groups evaluated. Red circles represent individuals not exposed to pesticides and blue circles refer to samples from exposed workers. (B) Clustering of the profile of miRNAs, instability damage, cytokinesis failure and cell death in the group exposed and not exposed to pesticides. The percentage of variance explained by each axis is reported in brackets.

3.10 Multivariate analysis of miRNAs differentially expressed in the exposed group

A multivariate analysis was conducted to examine the potential influence of various risk factors on miRNA expression patterns (Figure 10, Table S4). In sum, the only significant explanatory variable included in the final model for all 20 differentially expressed miRNAs was the exposure group variable.


miR-28-5p
3-Phenoxybenzoic acid
Smoking status
Groups
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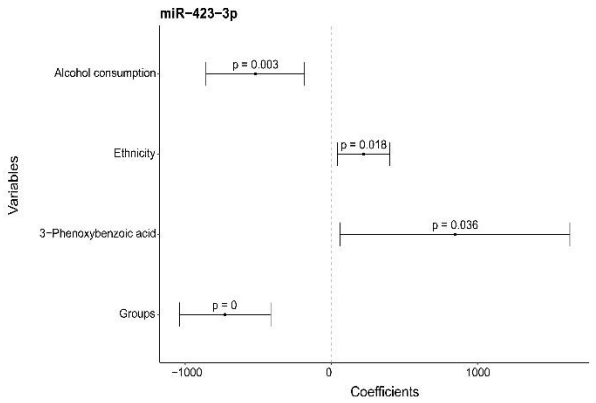
p = 0.084
p = 0.001
Variables
−500
0
500
Coefficients


miR-423-3p
Alcohol consumption
Ethnicity
3-Phenoxybenzoic acid
Groups
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p = 0.018
p = 0.036
p = 0
Variables
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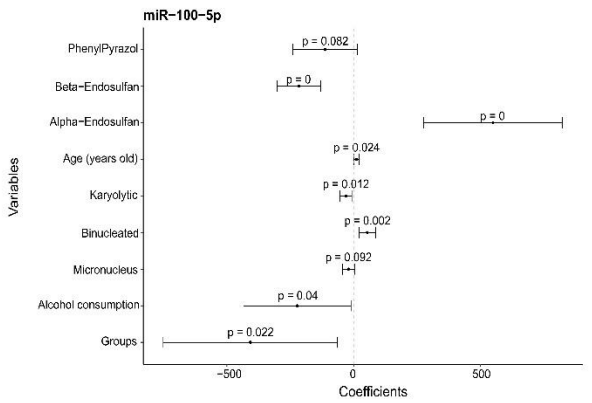
0
1000
Coefficients


miR-100-5p
PhenylPyrazol
Beta-Endosulfan
Alpha-Endosulfan
Age (years old)
Karyolytic
Binucleated
Micronucleus
Alcohol consumption
Groups
p = 0.082
p = 0
p = 0
p = 0.024
p = 0.012
p = 0.002
p = 0.092
p = 0.04
p = 0.022
Variables
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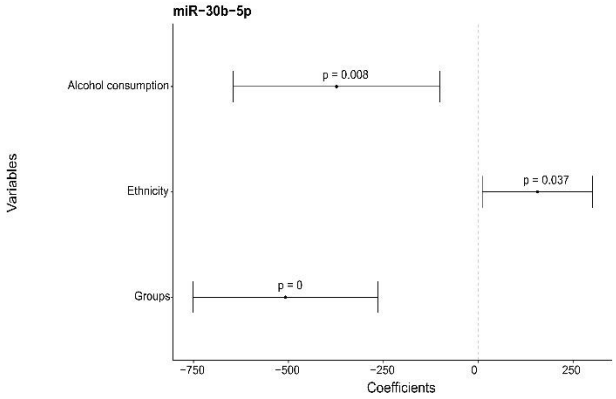
0
500
Coefficients


miR-30b-5p
Alcohol consumption
Ethnicity
Groups
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p = 0
Variables
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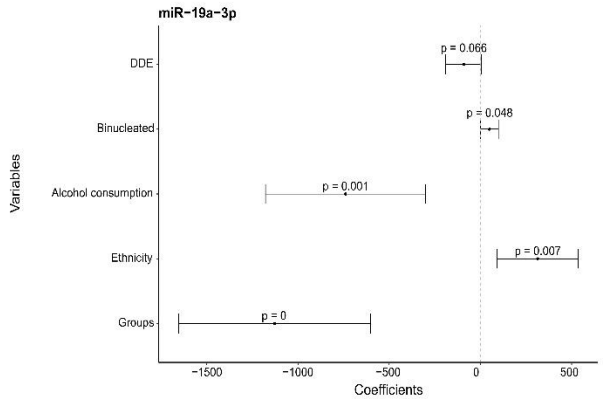
0
250
Coefficients


miR-19a-3p
DDE
Binucleated
Alcohol consumption
Ethnicity
Groups
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p = 0.001
p = 0.007
p = 0
Variables
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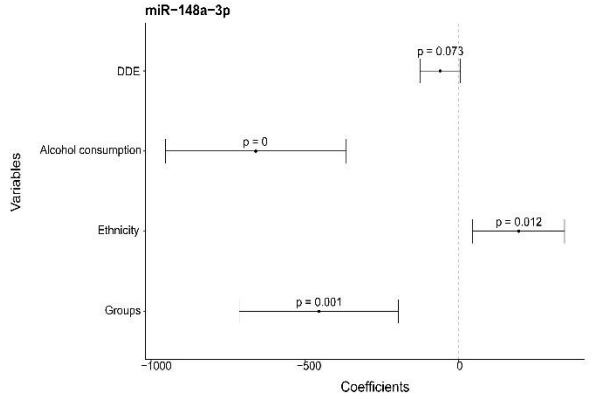
−1000
−500
0
500
Coefficients


miR-148a-3p
DDE
Alcohol consumption
Ethnicity
Groups
p = 0.073
p = 0
p = 0.012
p = 0.001
Variables
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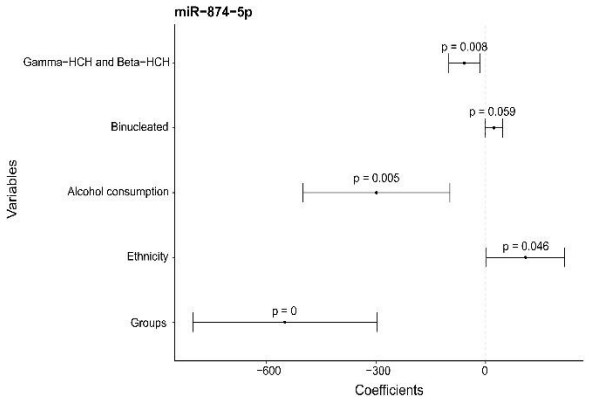
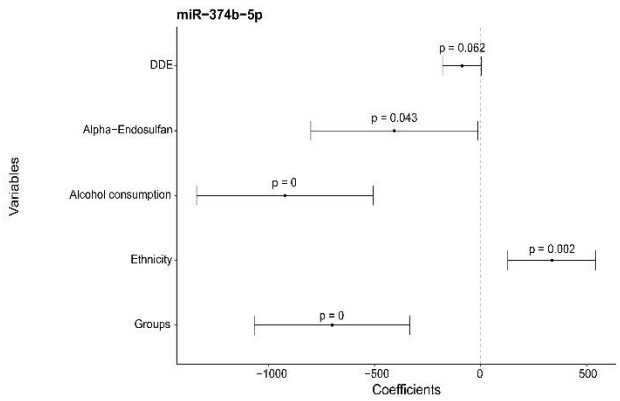
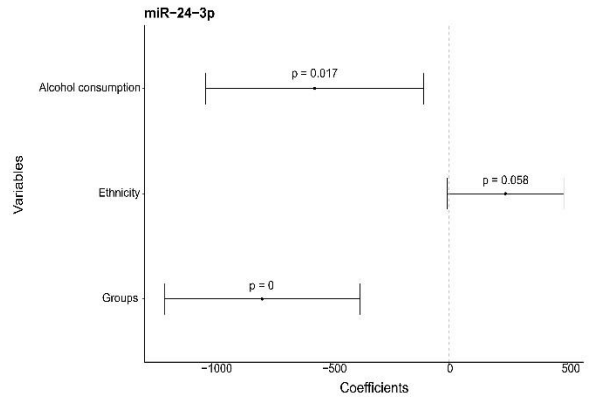
−500
0
Coefficients


miR-874-5p
Gamma-HCH and Beta-HCH
Binucleated
Alcohol consumption
Ethnicity
Groups
p = 0.008
p = 0.059
p = 0.005
p = 0.046
p = 0
Variables
−600
−300
0
Coefficients


miR-374b-5p
DDE
Alpha-Endosulfan
Alcohol consumption
Ethnicity
Groups
p = 0.062
p = 0.043
p = 0
p = 0.002
p = 0
Variables
−1000
−500
0
500
Coefficients


miR-24-3p
Alcohol consumption
Ethnicity
Groups
p = 0.017
p = 0.058
p = 0
Variables
−1000
−500
0
500
Coefficients

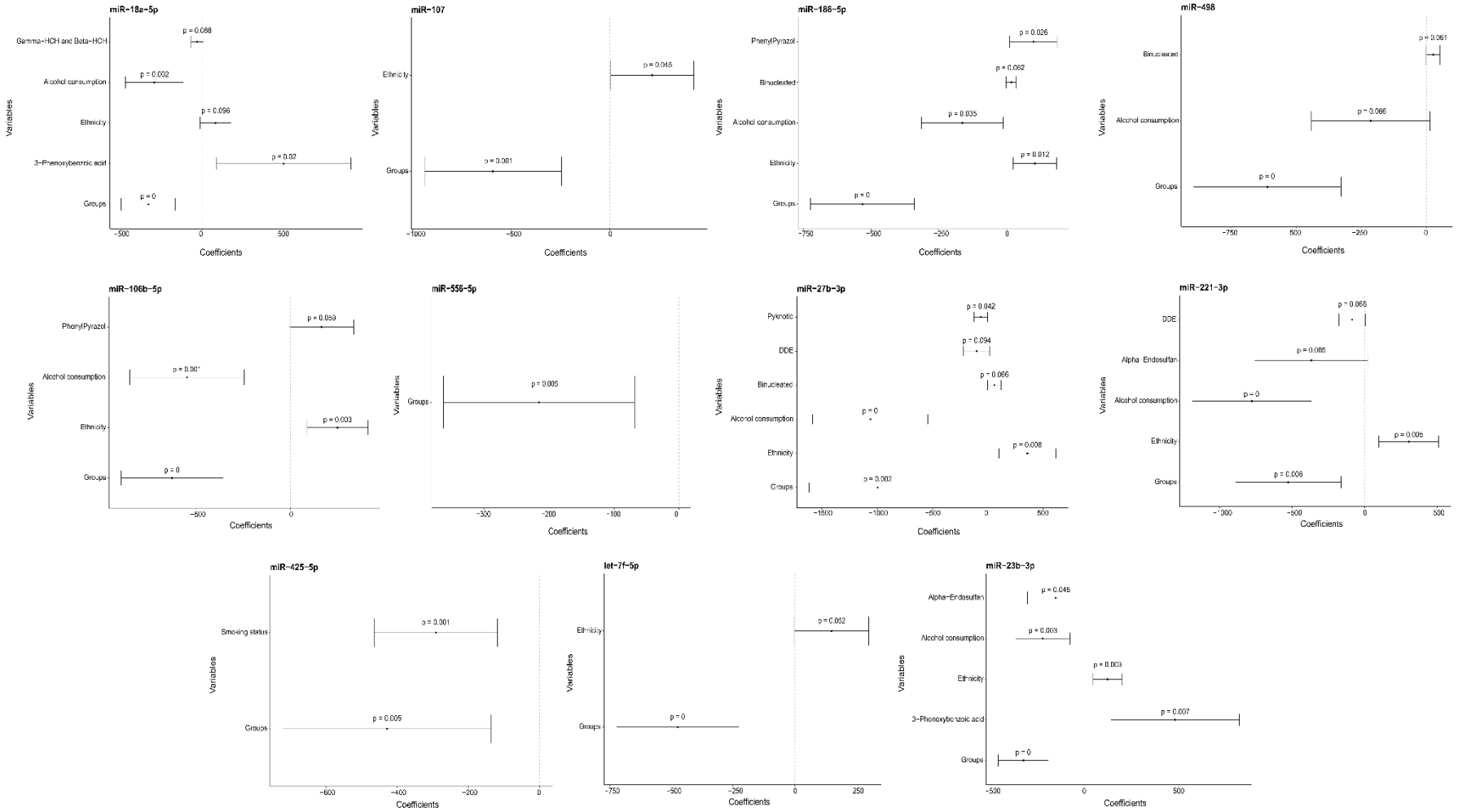


Figure 10. Multiple linear regression model with 95% confidence interval for the covariate coefficients and probability value for each respective response variable separated by matrix.

3.11 Prediction of target genes

A total of 1,004 validated miRNA target genes were predicted using the online tool mirDIP, with the selection criterion being genes with strong interactions (top 1%) with the differentially expressed miRNAs (Excel Table 1). It was observed that the miRNAs 556-5p and 874-5p did not exhibit any consensus targets among the five databases utilized. Furthermore, miR-498 exhibited an absence of any validated targets that were identified within mirDIP. Figure 11 depicts the interaction network between miRNAs and their target genes.

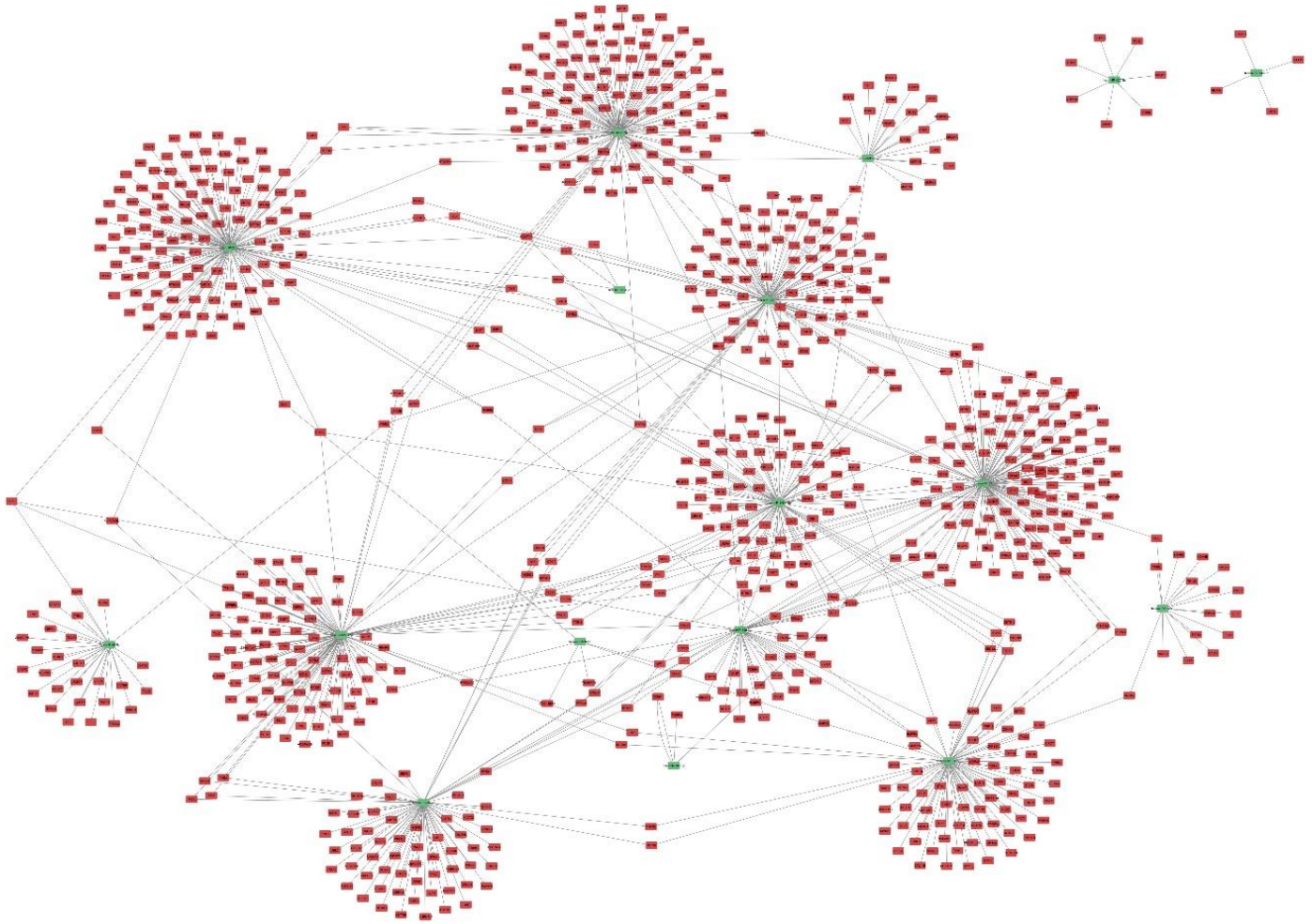
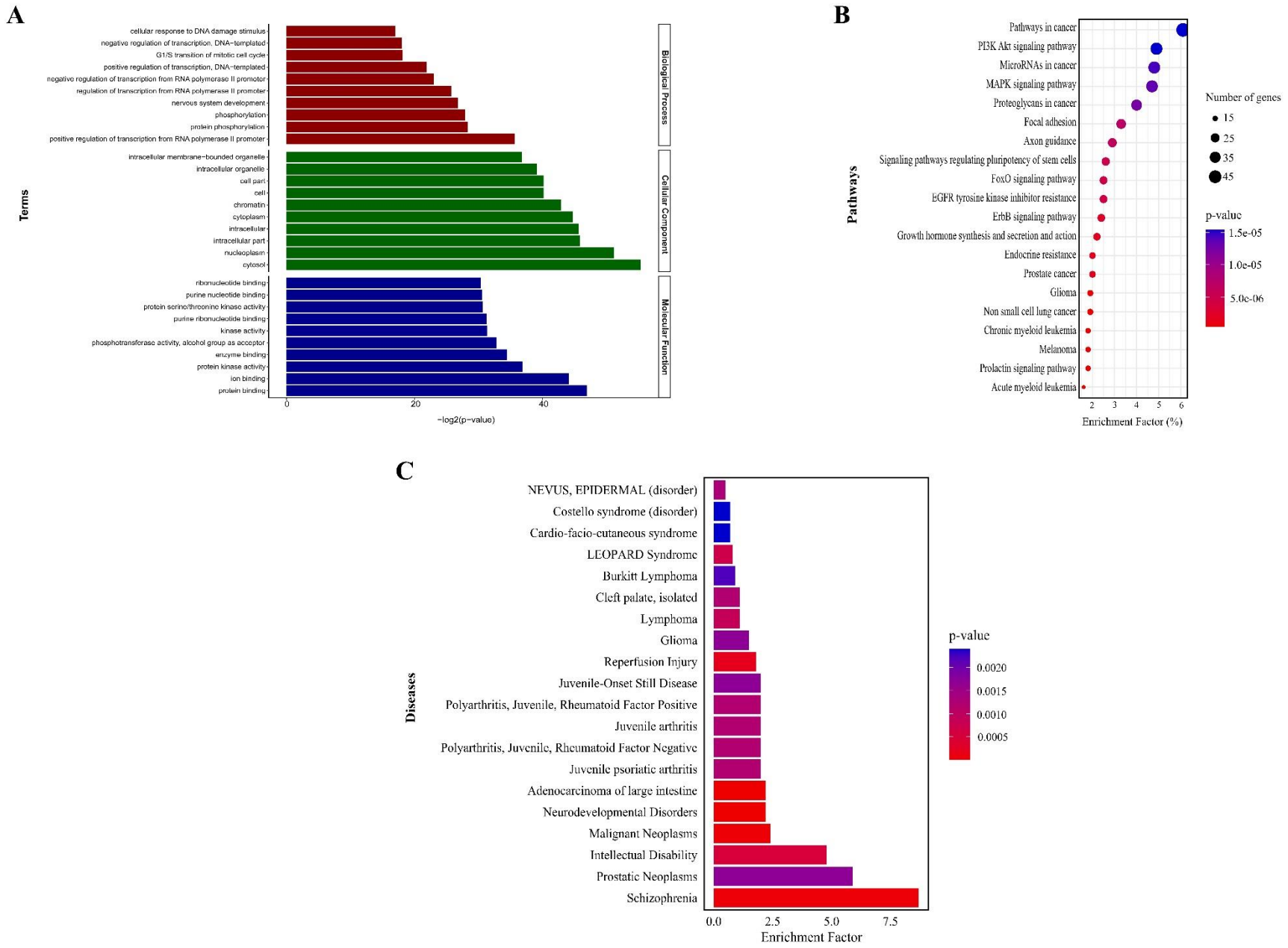


Figure 11. Interaction network between the 20 miRNAs differentially expressed between the pesticide-exposed and non-exposed groups and their target genes validated in databases. The red rectangles represent the target genes and the green rectangles represent the miRNAs negatively regulated in the pesticide-exposed group.

3.12 Functional enrichment analysis: Gene ontology

A Gene Ontology analysis was conducted to identify the potential functions of the target genes of differentially expressed miRNAs. The analysis identified 213 biological process terms, 131 cellular component terms, and 170 molecular function terms. The primary biological processes associated with miRNA target genes in pesticide-exposed workers were found to be related to transcription regulation (Figure 12A, Excel Table 2). The enriched terms for cellular components and molecular functions indicated associations with binding, protein activity, intracellular components, and protein-containing complexes (Figure 12A, Excel Tables 3 and 4). KEGG pathway enrichment analysis identified 91 enriched pathways for predicted target genes (Excel Table 5), with the top 20 significantly enriched pathways ($p < 0.0001$) including signaling, hormonal, and various cancer-related pathways (Figure 12B). Moreover, an analysis of the association between the target genes and disease revealed 107 diseases ($p < 0.05$), with the most prevalent being neurological disorders, arthritis, and cancer (Figure 12C and Excel Table 6).


A
cellular response to DNA damage stimulus
negative regulation of transcription, DNA−templated
G1/S transition of mitotic cell cycle
positive regulation of transcription, DNA−templated
negative regulation of transcription from RNA polymerase II promoter
regulation of transcription from RNA polymerase II promoter
nervous system development
phosphorylation
protein phosphorylation
positive regulation of transcription from RNA polymerase II promoter
intracellular membrane−bounded organelle
intracellular organelle
cell part
cell
chromatin
cytoplasm
intracellular
intracellular part
nucleoplasm
cytosol
ribonucleotide binding
purine nucleotide binding
protein serine/threonine kinase activity
purine ribonucleotide binding
kinase activity
phosphotransferase activity, alcohol group as acceptor
enzyme binding
protein kinase activity
ion binding
protein binding
Biological Process
Cellular Component
Molecular Function
Terms
0
20
40
−log2(p−value)
B
Pathways in cancer
PI3K Akt signaling pathway
MicroRNAs in cancer
MAPK signaling pathway
Proteoglycans in cancer
Focal adhesion
Axon guidance
Signaling pathways regulating pluripotency of stem cells
FoxO signaling pathway
EGFR tyrosine kinase inhibitor resistance
ErbB signaling pathway
Growth hormone synthesis and secretion and action
Endocrine resistance
Prostate cancer
Glioma
Non small cell lung cancer
Chronic myeloid leukemia
Melanoma
Prolactin signaling pathway
Acute myeloid leukemia
Pathways
2
3
4
5
6
Enrichment Factor (%)
Number of genes
15
25
35
45
p-value
1.5e-05
1.0e-05
5.0e-06
C
NEVUS, EPIDERMAL (disorder)
Costello syndrome (disorder)
Cardio-facio-cutaneous syndrome
LEOPARD Syndrome
Burkitt Lymphoma
Cleft palate, isolated
Lymphoma
Glioma
Reperfusion Injury
Juvenile-Onset Still Disease
Polyarthritis, Juvenile, Rheumatoid Factor Positive
Juvenile arthritis
Polyarthritis, Juvenile, Rheumatoid Factor Negative
Juvenile psoriatic arthritis
Adenocarcinoma of large intestine
Neurodevelopmental Disorders
Malignant Neoplasms
Intellectual Disability
Prostatic Neoplasms
Schizophrenia
Diseases
0.0
2.5
5.0
7.5
Enrichment Factor
p-value
0.0020
0.0015
0.0010
0.0005

=

Figure 12. Functional enrichment analysis of target genes of miRNAs as potential biomarkers of pesticide exposure. (A) TOP 5 gene ontology terms including biological processes, cellular components, and molecular functions annotated for the target genes. All terms have a p-value of less than 0.05. (B) Top 20 pathways based on the KEGG pathway database for the targets of negatively regulated miRNAs. All terms have a p-value of less than 0.05. The colors of the dots represent the p-value and the sizes of the dots represent the number of genes annotated in the pathway. (C) Top 20 diseases from the DisGeNET database associated with the predicted target genes for differentially expressed miRNAs. The colors of the bars represent the p-value.

4. Discussion

In this study, we characterized, for the first time, the changes in miRNA expression in the plasma of healthy Brazilian individuals occupationally exposed to pesticides. To this end, we employed a panel of 798 target miRNAs based on Nanostring technology. Furthermore, an increase in genomic instability, cytokinesis failure, and cell death was observed in the oral cells of individuals exposed to pesticides.

The presence of pesticide residues in the general population has been the subject of numerous studies conducted in a variety of locations across the globe. These studies have examined different chemical classes of active ingredients, many of which have been detected at concentrations as low as a few $ng.mL^{-1}$. These include legacy chemical compounds, such as organochlorines, which are known for their persistence and bioaccumulation, as well as excretable active ingredient residues (Birolli et al., 2024; Hakme et al., 2024). In this study, additional legacy pesticides were identified in the study participants, including alpha-endosulfan, beta-endosulfan, methoxychlor, lindane, and beta-HCH. Nevertheless, no statistically significant differences were identified between the groups.

The concentration of these pesticides can be compared with data observed in other epidemiological approaches. For example, alpha-endosulfan and beta-endosulfan were identified previously in Mexico (Rolando Adair et al., 2024) and India (Sharma et al., 2019). The HCH values observed in our study (2.13 $ng.mL^{-1}$) were also verified in Mexico and India (3.8 e 2.6 $ng.mL^{-1}$ γ-HCH, respectively) (Ghosh et al., 2018; Polanco Rodríguez et al., 2017). Additionally, our study identified the presence of methoxychlor (0.83 ± 1.62 $ng.mL^{-1}$), underscoring the importance of its evaluation in the context of legacy compounds, as observed in previous studies in Ethiopia (2.8 $ng.mL^{-1}$) and China (1.9 $ng.mL^{-1}$) (Luo et al., 2016; Mekonen et al., 2021).

The excretable compounds were also evaluated, but no significant differences were observed between the groups. The presence of PNP was identified at a concentration of 1.0 $ng.mL^{-1}$, 3-PBA at 0.8 $ng.mL^{-1}$, 2,4-dichlorophenol at 0.6 $ng.mL^{-1}$ and TCP at 0.8 $ng.mL^{-1}$. As these compounds are often analyzed in urine, a direct comparison with serum

determinations is not feasible. It is noteworthy that these compounds have been previously reported in studies with values comparable to those observed in the present study. For example, the concentration of PNP in the USA was found to be 0.7 $ng.mL^{-1}$ (Curl et al., 2021), while in Belgium and China, the concentrations of PBA were 1.0 $ng.mL^{-1}$ and 0.4 $ng.mL^{-1}$, respectively (Guo et al., 2017; Pirard et al., 2020). The concentrations of 2,4-dichlorophenol in the USA and Sweden were found to be 0.4 $ng.mL^{-1}$ and 0.1 $ng.mL^{-1}$, respectively (Goldberg et al., 2024; Norén et al., 2020). The concentrations of TCP in Iran and Europe was found to be 1.3 $ng.mL^{-1}$ and 2.4 $ng.mL^{-1}$, respectively (Brahmand et al., 2019; Govarts et al., 2023). The present study revealed a concentration of 1.7 $ng.mL^{-1}$ for FipSul. This pesticide has also been confirmed in serum (0.2 $ng.mL^{-1}$) and hair (2.3 $ng.g^{-1}$) in Chinese and French studies, respectively (Béranger et al., 2020; Shi et al., 2021). In order to evaluate pyraclostrobin, the compound PhenylPyrazol was employed as an exposure biomarker (0.3 $ng.mL^{-1}$), given that this analyte represents a primary product of pyraclostrobin metabolism in both animal and environmental systems (Birolli et al., 2020; WHO and FAO, 2019). In previous studies, pyraclostrobin metabolites were not selected for this purpose, which limited the basis for comparison in the present study.

The present study demonstrated that the prevalence of cell death (pyknosis and karyolysis), cytokinesis failure (binucleated cells), and genomic instability (MNC) was higher in the pesticide-exposed group of workers when compared to the non-exposed group. The results of the cytome assay indicated that the buccal cells of individuals with occupational exposure to pesticides exhibited elevated levels of genomic instability (MNC), cytokinesis failure (binucleated cells), and cell death (pyknosis and karyolysis) compared to the cells of individuals without such exposure. A comparable result was obtained in a preceding study conducted by our research group (dos Santos et al., 2022), individuals with exposure to pesticides exhibited higher frequencies of MNC, binucleated, karyorrhectic, and condensed chromatin cells when compared to the individuals in the non-exposed group. In addition to pesticide exposure, other substances have been identified as contributing to increased damage as detected by the cytome assay, these include coal, gasoline, PAH, and antineoplastic drugs (Hisamuddin et al., 2022; Rohr et al., 2013; Santos et al., 2020; Shahsavari et al., 2022). Furthermore, an increase in cytome assay endpoints has been observed in the context of aging and a number of diseases, including chronic kidney disease, COVID, and cancer (Pinto et al., 2021; Stopper et al., 2020).

A total of 30 miRNAs were identified as being significantly altered ($p < 0.005$) in the pesticide-exposed group when compared to the non-exposed group. Of the 30 differentially expressed miRNAs, 20 were identified as potential biomarkers of pesticide exposure based on their AUC value, specificity, and sensitivity. All 20 miRNAs demonstrated decreased expression in the group of workers exposed to pesticides.

A number of studies have examined the relationship between miRNAs and chemical substances. Similarly, the expression of miR-423-3p was found to be significantly reduced in workers exposed to diesel engine exhaust in comparison to non-exposed individuals (Hu et al., 2023). Additionally, the literature revealed that miR-19a-3p, 24-3p, and 28-5p demonstrated a notable decline in expression levels in individuals exposed to perfluoroalkyl substances, titanium, benz[a]pyrene-r-7,t-8,c-10-tetrahydrotetrol-albumin (BPDE-Alb) adducts. It is noteworthy that this decline has been observed in coke oven workers and in individuals with personal exposure to $PM_{2.5}$ (W. Chen et al., 2019; Deng et al., 2014, 2019; Mancini et al., 2020; Xu et al., 2020). In contrast with the findings of our study, previous research has indicated that miR-18a-5p and miR-425-5p levels were significantly upregulated in workers exposed to dust particles (Straumfors et al., 2020) and in individuals with personal exposure to $PM_{2.5}$ (Mancini et al., 2020), respectively.

In our study, an examination of the correlation between the 20 miRNAs and the pesticides quantified in the serum of the study participants yielded the result that 10 of the miRNAs were associated with individual pesticides. The expression levels of miR-423-3p in the group of exposed workers demonstrated a positive correlation with phenylpyrazole and alpha-endosulfan. Moreover, a notable positive correlation was observed between phenylpyrazole and the expression levels of miRNAs 221-3p, 107, and 374b-5p. The decreased expression of miR-221-3p observed in our study is also significantly downregulated in workers occupationally exposed to benzene (Liu et al., 2016). Furthermore, a negative correlation was observed between the pesticide TCP and the expression levels of four miRNAs, including miR-27b-3p, 30b-5p, 874-5p, and let-7f-5p. Additionally, our study revealed a positive correlation between the compound DDE and miR-23b-3p, while 2,4-D demonstrated a negative correlation with miR-556-5p.

To model the associations between miRNA levels and pesticide concentrations, as well as potential confounding factors, a variety of multivariate regression models were employed. The final models indicate that the only significant explanatory variable included in the final model for all 20 differentially expressed miRNAs was the exposure

group variable. In the final models for miRNAs 498, 556-5p, and let-7f-5p, the only significant variable was the aforementioned variable, which had a p-value less than 0.05. To date, no other study has demonstrated the associations observed in the present study. Among the few studies in the literature that have associated the expression of miRNAs with pesticide exposure, Weldon et al. (2016) demonstrated that miR-28-5p was present in 38% and 50% of urine samples from adults and children, respectively, who had been exposed to pesticides. Moreover, the miRNAs 425-5p, 106b-5p, and 30b-5p, identified in our study as exhibiting differential expression in the group of workers exposed to pesticides, were also observed by Krauskopf et al. (2017) in a population with verified exposure to persistent organic pollutants. The pesticides in question were hexachlorobenzene (HCB) and dichlorodiphenyltrichloroethane (DDT), two chemicals that have historically been used in agricultural pest control. In a related study, (Krauskopf et al., 2017)(2017) reported a significant association between miR-106b-5p and both DDT and HCB.

To identify the biological mechanisms underlying the differential expression of miRNAs, we conducted a functional analysis on the target genes of the 20 miRNAs that exhibited altered expression in the group of workers exposed to pesticides. The miRNAs were selected based on their capacity to differentiate between the pesticide-exposed and non-exposed groups, as determined by the receiver operating characteristic (ROC) curve. A total of 17 miRNAs were identified that exhibited biological functional interactions with 1,004 predicted mRNAs. The mRNAs were found to be enriched in 213 biological process terms, 131 cellular component terms, 170 molecular function terms, as well as 91 pathways and 107 diseases. The pathway enrichment analysis revealed that the target mRNAs were associated with multiple pathways, predominantly those related to the development of cancer and neurological diseases. This association was also observed in the DisGeNET analysis, which demonstrated that the primary diseases associated with the target genes were diseases affecting the neurological system and various types of cancer. These findings are consistent with those of previous studies that have demonstrated a correlation between pesticide exposure and the onset of neurological disorders (Chen et al., 2019; Dardiotis et al., 2019; de Graaf et al., 2022; Gama et al., 2022; Saeedi Saravi and Dehpour, 2016; Shrestha et al., 2020; Torres-Sánchez et al., 2023) and cancer (Alavanja et al., 2014; Benavente et al., 2020; Francisco et al., 2023; Gatto et al., 2021; Martin et al., 2018; Pardo et al., 2020; Remigio et al., 2024).

It is important to acknowledge the limitations of this study. The sample size of the study was relatively limited, and the study was conducted exclusively with male workers. Additionally, the results for the associations between exposure to pesticides and the individual quantification of the pesticides analyzed with the levels of miRNAs may be subject to multicollinearity of these compounds. It is also important to note that the possibility of other confounding factors or bias from undetermined contaminants in our study cannot be ruled out. The present study has several notable strengths. To the best of our knowledge, this is the first to comprehensively explore the epigenetic alterations induced by pesticide exposure in plasma-derived miRNAs from healthy individuals. The exposure factor was present in all final regression models for each miRNA under evaluation. Moreover, although the pesticides were quantified in serum, which limited the number of pesticides evaluated, our findings demonstrate that individuals still exhibited quantifiable levels of banned pesticides that are still present in their bodies. Another advantage was the use of a panel comprising 798 target miRNAs, which enabled a more comprehensive assessment of the miRNA expression profile associated with pesticide exposure through the use of Nanostring technology. Twenty miRNAs were identified as potential biomarkers of exposure, as they demonstrated discriminatory power between the pesticide-exposed and non-exposed groups in ROC curve analysis. Furthermore, we were able to predict genes that were likely to be modulated by the miRNAs, as well as predict the potential biological functions, pathways, and diseases associated with the target genes of the miRNAs identified as potential biomarkers of pesticide exposure.

5. Conclusion

In conclusion, our findings provide direct evidence that alterations in plasma miRNA expression are associated with pesticide exposure. Twenty miRNAs were identified as potential biomarkers of pesticide exposure, all of which exhibited decreased expression in the group of exposed workers. Our findings contribute new insights into the biological pathways underlying the effects of pesticide exposure and illustrate the pivotal role of miRNAs in the pathogenesis of exposure-related diseases, particularly those affecting the neurological system and various types of cancer. In general, there is a limited amount of data available regarding the correlation between pesticide exposure and miRNAs. Consequently, our data can inform the implementation of preventive environmental monitoring and diagnosis. Nevertheless, further prospective cohort studies

with larger sample sizes are required to gain a deeper understanding of the mechanisms underlying pesticide-associated changes in plasma miRNAs.

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Author Contributions

Luiza Flavia Veiga Francisco: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Writing – original draft. Willian Garcia Birolli: Methodology; Investigation, Data curation; Formal analysis. Ana Julia Aguiar de Freitas: Methodology; Investigation, Data curation; Formal analysis. Welinton Hirai: Methodology; Data curation; Formal analysis. Paula Rohr: Methodology; Data curation; Formal analysis. Caroline Rocha Nunes: Methodology. Álvaro José dos Santos Neto: Writing – review & editing. Fernando Barbosa Junior: Writing – review & editing. Márcia Maria Chiquitelli Marques: Conceptualization; Writing – review & editing. Henrique C. S. Silveira: Conceptualization; Methodology; Supervision; Writing – review & editing. All authors read and approved the final manuscript.

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Declaration of interests

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 Statement:

Due to the sensitive nature of the questions asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

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CONCLUSÕES

Como conclusão dessa tese, nossa revisão sistemática com meta-análise, forneceu evidências da associação entre exposições ocupacionais e o risco de desenvolvimento de Linfoma não Hodgkin por meio de uma avaliação detalhada de estudos epidemiológicos. Demonstramos que o risco de LNH é aumentado em pessoas ocupacionalmente expostas a substâncias químicas, como os pesticidas, ou que exercem algum tipo de trabalho específico, porém, ainda faltam dados sobre a associação de LNH e compostos químicos específicos. Além disso, foi verificado que uma das principais classes de risco para o desenvolvimento de LNH foi o trabalhador rural. Nossos achados podem auxiliar com informações para tomadas de decisão em saúde pública e práticas sobre determinadas atividades de trabalho e uso de compostos químicos. Evidências sobre a associação de classes específicas de compostos químicos e classes de trabalho com o desenvolvimento de LNH em amostras biológicas, ainda são limitadas, assim, estudos futuros na avaliação de mecanismos, com mensurações das exposições e avaliação dos efeitos biológicos e moleculares associados com o risco de LNH são necessários.

Considerando os resultados anteriores obtidos em nossa revisão sistemática, realizamos estudos moleculares com indivíduos expostos a pesticidas. Em relação aos dados genotóxicos e moleculares obtidos do grupo de indivíduos saudáveis expostos e não expostos a agrotóxicos, os resultados demonstraram que as células dos indivíduos saudáveis expostos apresentaram maior genotoxicidade, falha na citocinese e citotoxicidade do que as células dos indivíduos não expostos. Além disso, podemos observar que a exposição causou alteração epigenética nestes indivíduos por meio da alteração do perfil de miRNAs. A exposição aos pesticidas causou regulação negativa de muitos miRNAs no grupo de indivíduos expostos. Os genes- alvos destes miRNAs estão associados a diversas doenças, principalmente doenças neurológicas e diversos tipos de câncer. Além disso, os resultados moleculares obtidos dos pacientes com LLC e MM reforçaram que a exposição aos pesticidas causa alteração epigenética em indivíduos com doença expostos ocupacionalmente. No grupo de pacientes expostos também foi verificado uma diminuição na expressão dos miRNAs, os quais também demonstraram genes- alvos preditos relacionados com doenças neurológicas e câncer. Assim, pode-se inferir que os resultados obtidos demonstraram estar diretamente relacionados ao

fator exposição e que os indivíduos expostos estão mais suscetíveis a riscos na saúde do que a população não exposta a estes compostos. Além disso, podemos salientar que a identificação de biomarcadores epigenéticos em indivíduos expostos aos pesticidas, como os miRNAs, podem ter um impacto considerável na prevenção do desenvolvimento de múltiplas doenças, o que pode contribuir no futuro com a medicina ambiental de precisão. Porém, mais estudos são necessários para identificar o papel das alterações ao longo das vias, determinando os efeitos dos pesticidas na prevenção de doenças.

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ANEXOS

Anexo A - Parecer consubstanciado do Comitê de Ética e Pesquisa (CEP): Avaliação de biomarcadores em indivíduos ocupacionalmente expostos aos agrotóxicos: uma abordagem exposômica.

DADOS DA EMENDA

Título da Pesquisa: Avaliação de biomarcadores em indivíduos ocupacionalmente expostos aos agrotóxicos: uma abordagem exposômica

Pesquisador: Henrique César Santejo Silveira

Área Temática: Genética Humana:

(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 5

CAAE: 00270418.2.0000.5437

Instituição Proponente: Fundação Pio XII

Patrocinador Principal: Fundação Pio XII

DADOS DO PARECER

Número do Parecer: 4.963.507

Apresentação do Projeto:

As informações elencadas nos campos denominados "apresentação do projeto", "objetivos" e "avaliação dos riscos e benefícios" foram retiradas do documento intitulado "PB_INFORMAÇÕES_BÁSICAS_1764880_E1.pdf" (submetido na Plataforma Brasil em 24/08/2021).

Resumo:
Nos últimos cinquenta anos, a agricultura sofreu uma profunda mudança com o crescimento massivo do uso de agrotóxicos para melhorar a produção e proteção da colheita, assim como a qualidade e preservação do alimento. O Brasil é um dos maiores consumidores de agrotóxicos do mundo. Com o aumento da utilização de agrotóxicos nos últimos anos no país, aumenta também o risco de exposição de trabalhadores como também da população através da contaminação do meio ambiente, da água e alimentos. Devido à sua toxicidade intrínseca, esses agrotóxicos podem causar alguns efeitos deletérios como, alergias; distúrbios gastrintestinais, respiratórios, endócrinos, reprodutivos e neurológicos; e até mesmo câncer. Sendo assim, o objetivo desse projeto é analisar o comprimento dos telômeros, perfil de miRNAs e metilação do DNA como potenciais biomarcadores para associação do potencial risco de desenvolvimento de câncer em trabalhadores rurais expostos aos agrotóxicos. Trata - se de um estudo observacional transversal que analisará grupos de indivíduos expostos e não expostos que serão recrutados nos municípios que compõem a DRS-Barretos. Serão analisados

400 participantes na faixa etária de 21 a 65 anos, divididos em quatro grupos; Grupo 1: Indivíduos não expostos aos agrotóxicos, Grupo 2: Indivíduos expostos aos agrotóxicos, Grupo 3: Pacientes com câncer e que tiveram exposição aos agrotóxicos durante a vida ocupacional (expostos), Grupo 4: Pacientes com câncer que não tiveram exposição (não exposto) aos agrotóxicos. Esse estudo visa fundamentar a importância da utilização de biomarcadores como os telômero, miRNAs e metilação do DNA para a avaliação da instabilidade genômica e da alteração epigenética em relação a exposição aos agrotóxicos e o desenvolvimento de câncer.

Hipótese:
Acredita-se que esse estudo possa fundamentar a importância da utilização de biomarcadores para a avaliação de populações expostas aos agrotóxicos.

Metodologia Proposta:
4.1. Delineamento do estudoTrata-se de um estudo observacional transversal.4.2. Local de Estudo O estudo será realizado no Hospital de Câncer de Barretos no Centro de Pesquisa em Oncologia Molecular, bem como, os grupos de indivíduos expostos e não expostos que serão recrutados nos municípios que compõem a DRS-Barretos. 4.3. População de Estudo Os sujeitos da pesquisa deverão responder a um questionário referente às características sócio demográfico, rotina ocupacional e a saúde geral. Todos os entrevistadores serão treinados para a aplicação do questionário. O questionário que será utilizado foi adaptado em parte de Boffeta et al. 1999 e Sartor, 2003 e também no Agricultural Health Study (https://aghealth.nih.gov/). Este projeto utilizará quatro grupos para as análises, sendo estes: 1) Indivíduos não expostos aos agrotóxicos, constituindo o grupo controle.2) Trabalhadores rurais expostos aos agrotóxicos, grupo exposto.3) Um grupo de pacientes com câncer e que tiveram exposição aos agrotóxicos durante a vida ocupacional (expostos).4) Um grupo com pacientes com câncer que não tiveram exposição (não exposto) aos agrotóxicos e também a nenhum agente listado nas diretrizes do Cancer relacionado ao trabalho (INCA, 2012).O material Biológico que será utilizado nos dois primeiros grupos está estocado no Biobanco do HCB e foi coletado no projeto cujo intitulado “Avaliação de risco e conscientização do câncer ocupacional em trabalhadores rurais na região de Barretos” o qual foi obtido aprovação para análise do comprimento telomérico. Para os grupos de pacientes com câncer o material biológico será utilizado do estudo “Questionário Simplificado de Rastreio de câncer ocupacional (QSR) (CEP - 51644015.8.0000.5437), o qual também já obteve aprovação para avaliação do comprimento telomérico. Para a escolha dos tumores dos participantes com câncer serão avaliados os agrotóxicos mais frequentes do grupo de indivíduos saudáveis expostos e avaliada a ligação dos princípios ativos com os tipos tumorais. Todos os participantes com câncer serão participantes do QSR serão entrevistados pela equipe do projeto com o questionário referente ao histórico de utilização de agrotóxicos, já utilizado para o grupo de indivíduos saudáveis expostos conforme o projeto citado acima. 4.4. Cálculo do tamanho amostral. Para o cálculo amostral foi realizado o teste de comparação de médias através da relação entre grupos expostos e não expostos a pesticidas considerando o trabalho Kahl et al., 2016. O programa utilizado para realização do tamanho amostral foi GPower 3.0.10, levando-se em conta o poder de teste 0,8 e uma significância 0.05. Sendo assim, foi encontrado um número de 100 trabalhadores rurais e 100 indivíduos do grupo controle considerado ideal para avaliação dos biomarcadores nesta população. Para o grupo de pacientes com câncer foi estipulado por conveniência o mesmo número do grupo de

trabalhadores rurais. Para análise do comprimento absoluto dos telômeros será empregado o método proposto por O´Callaghan e Fenech, 2011. Para isto, utilizaremos a metodologia de PCR em tempo Real. A análise do perfil de miRNAs será realizado pelo ensaio de NanoString nCounter, utilizado o nCounter® miRNA Expression Assays (NanoString Technologies). Enquanto que a avaliação da metilação do DNA será realizada pelo método de Reduced-representation bisulfite sequencing (RRBS).

Critério de Inclusão:
Para o grupo de indivíduos expostos e não expostos todos os envolvidos devem estar residindo na mesma área do estudo por pelo menos 1 ano. Serão considerados para os grupos indivíduos com idade superior a 21 anos e abaixo dos 65 anos de idade. Para o grupo de indivíduos expostos serão incluídos no estudo trabalhadores rurais preferencialmente com exposição aos agrotóxicos pelo menos por 5 anos. Além disso, para o grupo de indivíduos não expostos serão selecionados participantes sem histórico de contato com os agrotóxicos ou com outros agentes considerados carcinogênicos. Para o grupo de pacientes com câncer serão incluídos os indivíduos expostos e não expostos aos agrotóxicos de acordo com o estudo QSR. Os grupos de pacientes com câncer serão pareados com o grupo de indivíduos expostos de acordo com idade, sexo, hábito de fumar, tempo de exposição aos agrotóxicos e local de origem.
Critério de Exclusão:
Para os grupos de estudo não serão selecionados indivíduos abaixo de 21 anos, fumantes, usuários de drogas, trabalhadores com presença de
doenças infecciosas ou doenças crônicas (como as doenças autoimunes), expostos a outros agentes, como raios X e xilol. Indivíduos com histórico de câncer ao longo da vida não serão considerados no estudo. Para os pacientes com câncer não serão incluídos os que tenham mais de uma exposição ao longo da vida, serão priorizados indivíduos com exposição somente aos agrotóxicos.

Objetivo da Pesquisa:
Objetivo Primário:
Análise de biomarcadores para associação do potencial risco de desenvolvimento de câncer em trabalhadores rurais expostos aos agrotóxicos.

Objetivo Secundário:
- Implementação e otimização da metodologia do comprimento relativo dos telômeros nos leucócitos de indivíduos expostos aos agrotóxicos;
- Avaliação do comprimento dos telômeros em um grupo de indivíduos saudáveis expostos e não expostos aos agrotóxicos.
- Realizar avaliação dos telômeros em pacientes com câncer expostos e não expostos aos agrotóxicos;
- Determinar o perfil de expressão de miRNAs em pacientes com câncer expostos e não expostos aos agrotóxicos;
- Avaliar o perfil de metilação do DNA em pacientes com câncer expostos e não expostos aos agrotóxicos;
- Correlacionar os dados demográficos e o histórico de exposição aos agrotóxicos com o comprimento relativo do telômero, perfil de miRNA e níveis da metilação do DNA entre os grupos.

Avaliação dos Riscos e Benefícios:
De acordo com os pesquisadores:
Riscos:
O projeto é de baixo risco para os participantes de pesquisa, uma vez que não causa constrangimento na aplicação do questionário na população de estudo. O que pode ocorrer é a quebra acidental do sigilo das informações dos participantes. A equipe do projeto tomará todas as precauções para que isso não ocorra.
Benefícios:
Não há benefício individual direto ou compensação financeira ao participantes do estudo. Esse estudo poderá auxiliar na caracterização das populações expostas aos agrotóxicos e que podem influenciar no desenvolvimento de câncer. Assim, será possível, a elaboração de políticas de saúde que propiciem a melhoria das condições de trabalho e qualidade de vida da população.

Comentários e Considerações sobre a Pesquisa:
RESPOSTAS REFERENTES ÀS PENDÊNCIAS EMITIDAS NO PARECER N°4.908.464, submetidos pelo CEP em 16/08/2021.
1) No documento intitulado PB_INFORMAÇÕES_BÁSICAS_1764880_E1.pdf e ProjetoFinal_CEP.docx o cronograma encontra-se desatualizado. Solicita-se atualização.

RESPOSTA PESQUISADOR: O cronograma foi atualizado.
Qual documento e item/página é possível localizar as alterações/correções realizadas: A alteração pode ser encontrada na Plataforma Brasil e ProjetoFinal_CEP (item 8.Cronograma página 23).

ANÁLISE CEP: PENDÊNCIA ATENDIDA.
2) O projeto original traz em seu cronograma a duração de 4 semestres a se iniciar em 2018, entretanto nenhum relatório semestral bem como o relatório final foram localizados na Plataforma Brasil demonstrando fatos relevantes e resultados parciais de seu desenvolvimento, conforme previsto na Resolução 466/2012. Ainda, a previsão de término da pesquisa seria em 2020, porém nenhuma emenda solicitando a extensão de prazo foi localizado na Plataforma Brasil, conforme previsto na Norma Operacional nº 001/2013. Portanto, solicita-se apresentação do relatório bem como a atualização do status do projeto.

RESPOSTA PESQUISADOR: O relatório com os resultados obtidos até o momento foram anexados na Plataforma Brasil. A extensão do prazo foi solicitada juntamente com as demais alterações na emenda apresentada.
Qual documento e item/página é possível localizar as alterações/correções realizadas: O relatório com os resultados foi anexado na Plataforma Brasil (Relatório_Resultados). O pedido de extensão do prazo pode ser encontrado na Emenda_CEP (item h).
ANÁLISE CEP: PENDÊNCIA ATENDIDA.

Considerações sobre os Termos de apresentação obrigatória:
Todos os termos foram adequadamente apresentados.

Recomendações:
Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:
Sem óbices éticos.
Considerações Finais a critério do CEP:
Enquanto isso utilizar:
O Comitê de Ética em Pesquisa da Fundação Pio XII - Hospital de Câncer de Barretos analisou o(s) seguinte(s) documento(s) do projeto 1676/2018, e:
- Aprovou a emenda ao estudo, submetida em 24/08/2021;

Após análise do(s) documento(s) supracitado(s), o Comitê faz a seguinte recomendação:
(x) O Estudo deve Continuar;
( ) O Estudo dever ser Interrompido;
( ) O Estudo está Finalizado;
( ) Solicita-se Esclarecimento.

Ressalta-se que cabe ao pesquisador responsável encaminhar os relatórios parciais e final da pesquisa, por meio da Plataforma Brasil, via notificação do tipo "relatório" para que sejam devidamente apreciados no CEP, conforme Norma Operacional nº001/13, item XI.2.d.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_1764880_E1.pdf	24/08/2021 08:36:58		Aceito
Outros	Carta_de_resposta.pdf	24/08/2021 08:35:43	Luiza Flavia Veiga Francisco	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoFinal_CEP.docx	07/08/2021 17:48:05	Luiza Flavia Veiga Francisco	Aceito
Outros	Relatorio_Resultados.pdf	07/08/2021 17:46:26	Luiza Flavia Veiga Francisco	Aceito
Outros	Emenda_CEP.pdf	07/08/2021 17:43:25	Luiza Flavia Veiga Francisco	Aceito
Outros	Emenda_CEP.docx	07/08/2021 17:43:04	Luiza Flavia Veiga Francisco	Aceito
Folha de Rosto	Folha_de_Rosto_Atualizada.pdf	14/07/2021 17:02:42	Luiza Flavia Veiga Francisco	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_final.docx	14/07/2021 16:55:14	Luiza Flavia Veiga Francisco	Aceito
Outros	Questionario_Sociodemografico.pdf	17/10/2018 09:20:09	Henrique César Santejo Silveira	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEHCBFINAL16102018final.docx	17/10/2018 09:19:52	Henrique César Santejo Silveira	Aceito

Outros	Cartarespostapendencias16102018.docx	17/10/2018 09:19:37	Henrique César Santejo Silveira	Aceito
Outros	Cartarespostapendencias16102018.pdf	17/10/2018 09:18:01	Henrique César Santejo Silveira	Aceito
Outros	MabincadastrodeprojetoIsabela.pdf	03/10/2018 15:18:56	Henrique César Santejo Silveira	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoFinal.docx	03/10/2018 13:54:20	ISABELA MARIA CAMPANELLI DOS SANTOS	Aceito
Outros	Declaracaoorcamentariaedeviabilidade.jpeg	25/09/2018 11:21:45	ISABELA MARIA CAMPANELLI DOS SANTOS	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLEHCBFINAL.docx	14/09/2018 09:45:37	ISABELA MARIA CAMPANELLI DOS SANTOS	Aceito
Outros	Declaracaoderesponsabilidadedopesquisador.jpeg	21/08/2018 13:27:58	ISABELA MARIA CAMPANELLI DOS SANTOS	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BARRETOS, 10 de Setembro de 2021

Assinado por: Thiago Buosi Silva (Coordenador)

Anexo B – Parecer consubstanciado do Comitê de Ética e Pesquisa (CEP): questionário simplificado de rastreio de câncer ocupacional (QSR) Como possível ferramenta para a identificação de neoplasias relacionadas ao trabalho.

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: QUESTIONÁRIO SIMPLIFICADO DE RASTREIO DE CÂNCER OCUPACIONAL (QSR) COMO POSSÍVEL FERRAMENTA PARA A IDENTIFICAÇÃO DE NEOPLASIAS RELACIONADAS AO TRABALHO

Pesquisador: Fabiana de Lima Vazquez

Área Temática:

Versão: 2

CAAE: 51644015.8.0000.5437

Instituição Proponente: Fundação Pio XII

Patrocinador Principal: Fundação Pio XII

DADOS DO PARECER

Número do Parecer: 1.392.203

Apresentação do Projeto:
Estudo transversal observacional prospectivo
Os critérios de inclusão do estudo serão: homens e mulheres com 30 anos ou mais, trabalhadores, empregados ou não, aposentados ou não, com diagnóstico recente de câncer de pele não melanoma, melanoma, pulmão, mesotelioma, bexiga, cavidade nasal e seios paranasais, cavidade oral, faringe e laringe, leucemias e mielodisplasias, mieloma múltiplo, linfoma de Hodgkin, estômago e esôfago, fígado, pâncreas, mama e sistema nervoso central, que estiverem dispostos a participar do estudo e que foram diagnosticados no mês do recrutamento. Os participantes deverão ser legalmente competentes para dar consentimento para entrar do estudo. Não serão incluídas no estudo homens ou mulheres que nunca trabalharam e que se recusarem a assinar o termo de consentimento.
Amostra de conveniência. O questionário será aplicado a todos os pacientes com câncer que pode ser relacionado ao trabalho ou ocupação (câncer de pele não melanoma, melanoma, pulmão, mesotelioma, bexiga, cavidade nasal e seios paranasais, cavidade oral, faringe e laringe, leucemias e mielodisplasias, mieloma múltiplo, linfoma de Hodgkin, estômago e esôfago, fígado, pâncreas, mama e sistema nervoso central), diagnosticados de abril de 2016 a maio de 2017 no HCB e que quiserem participar do estudo. A estimativa do número de novos casos/ano teve como base o Registro Hospitalar do HC Barretos. Foram 6636 novos casos no ano de 2014, considerando-se a faixa etária acima de 30 anos, foi estimado uma amostra de conveniência de 5000 participantes.

Objetivo da Pesquisa:
Objetivo principal:
-Verificar a viabilidade de aplicar um questionário simplificado que obtenha informações sobre exposição e ocupação, e verificar se essas informações permitem uma análise posterior elucidativa para implementar na prática clínica a ficha de notificação compulsória de câncer ocupacional e desencadear uma investigação e ações de vigilância nos ambientes e processos de trabalho.
Objetivos específicos:
-Avaliar a aplicabilidade de um questionário simplificado (QSR) na estratégia de rastreio de Câncer Ocupacional em pacientes com diagnóstico de neoplasia, no Hospital de Câncer de Barretos;
-Mensurar o tempo de aplicação do QSR;
-Realizar análise descritiva das informações contidas no formulário de notificação compulsória dos casos identificados pelo QSR como possivelmente relacionados à atividade ocupacional.
-Quantificar os casos de Câncer Ocupacional notificados após a aplicação do questionário.

Avaliação dos Riscos e Benefícios:
O risco principal para esse estudo é a quebra acidental de sigilo, que os pesquisadores cuidadosamente vão procurar minimizar em relação à privacidade, armazenamento de dados e confidencialidade.
Os benefícios para o participante e / ou Sociedade: O benefício para o participante será a avaliação de história laboral de exposição a riscos, podendo ser passível de notificação caso essa possibilidade ocorra. O benefício potencial do estudo para a sociedade é que o estudo proposto forneça ferramentas para que a notificação de câncer ocupacional seja viável e que mais trabalhadores sejam beneficiados e que medidas de prevenção sejam implementadas para que novos casos de câncer ocupacional possam ser evitados.

Comentários e Considerações sobre a Pesquisa:
Sem comentários e considerações adicionais.

Considerações sobre os Termos de apresentação obrigatória:
Todos os documentos foram apresentados de forma adequada.

Recomendações:
Conclusões ou Pendências e Lista de Inadequações:
1. O item "O QUE ACONTECERÁ COMIGO DURANTE O ESTUDO?" trás informações apenas sobre o preenchimento de um questionário simplificado sobre a sua profissão e apenas isso. Este item deve apresentar também uma estimativa de tempo previsto para a conclusão do mesmo. Deve-se esclarecer também quanto à possibilidade de se realizar notificação ao SINAN e a emissão de CAT conforme previsto no projeto. Na página 13 item 4.8.3 Notificação ao SINAN: Lê-se “Os casos de câncer com possível relação com exposição ocupacional serão notificados ao SINAN (Sistema Nacional de Agravos de Notificação) e emitida a CAT (Comunicação de Acidente de Trabalho) para os trabalhadores segurados pela Previdência Social”.
RESPOSTA: Foi modificado no projeto (em vermelho): O QUE ACONTECERÁO QUE ACONTECERÁ COMIGO DURANTE O ESTUDO?

Você irá responder um questionário simplificado sobre a sua profissão. Esse questionário tem a duração de menos de 5 minutos. Caso seja identificada alguma possibilidade de relação do seu tipo de câncer com o seu trabalho ou ocupação (atual ou no passado), será preenchida uma ficha de notificação obrigatória do Instituto Nacional de Câncer /Ministério da Saúde que se chama SINAN (Sistema Nacional de Agravos de Notificação) que se encarregará de emitir o CAT (Comunicação de Acidente de Trabalho) para os trabalhadores segurados pela Previdência Social.
ANÁLISE CEP: PENDÊNCIA ATENDIDA.

Considerações Finais a critério do CEP:
O Comitê de Ética em Pesquisa da Fundação Pio XII – Hospital do Câncer de Barretos de acordo com as atribuições definidas na Resolução CNS 466/2012 e na Norma Operacional Nº 001/2013 do CNS, e após a análise das respostas as pendências emitidas, manifesta-se pela APROVAÇÃO do projeto de pesquisa proposto.

Solicitamos que sejam encaminhados ao CEP:
1. Relatórios semestrais, sendo o primeiro previsto para 21/07/2016.
2. Comunicar toda e qualquer alteração do Projeto e Termo de Consentimento Livre e Esclarecido. Nestas circunstâncias a inclusão de participantes deve ser temporariamente interrompida até a aprovação do Comitê de Ética em Pesquisa.
3. Comunicar imediatamente ao Comitê qualquer Evento Adverso Grave ocorrido durante o desenvolvimento do estudo.
4. Para projetos que utilizam amostras criopreservadas, procurar o BIOBANCO para início do processamento.
5. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos, após conclusão da pesquisa, para possível auditoria dos órgãos competentes.
6. Este projeto está cadastrado no CEP-HCB sob o número 1056/2015.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_634370.pdf	11/01/2016 15:33:16		Aceito
Outros	CARTA_DE_RESPOSTA.pdf	11/01/2016 15:30:01	Larissa Cristina Ferreira	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	3_TCLE_JH_FV_review.doc	11/01/2016 15:06:19	Fabiana de Lima Vazquez	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_epi_9.docx	11/01/2016 15:05:58	Fabiana de Lima Vazquez	Aceito
Outros	declaracao_fonte_financiamento.pdf	04/12/2015 16:03:46	Larissa Cristina Ferreira	Aceito
Outros	Cadastro_Projeto.pdf	04/12/2015 10:08:59	Larissa Cristina Ferreira	Aceito
Outros	MABIN.pdf	04/12/2015 10:07:33	Larissa Cristina Ferreira	Aceito

Declaração de Pesquisadores	Declaracao_Resp_Pesquisador.pdf	04/12/2015 10:06:12	Larissa Cristina Ferreira	Aceito
Outros	Declaracao_Ciencia_Autorizacao_Estudo.pdf	04/12/2015 10:00:51	Larissa Cristina Ferreira	Aceito
Folha de Rosto	Folha_de_Rosto.pdf	04/12/2015 08:04:39	Larissa Cristina Ferreira	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BARRETOS, 21 de Janeiro de 2016

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Assinado por: Thiago Buosi Silva (Coordenador)