



Effects of pesticide exposure on the expression of selected genes in normal and cancer samples: Identification of predictive biomarkers for risk assessment

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ABSTRACT

Pesticides pivotal in controlling pests, can represent a threat for human health. Regulatory agencies constantly monitor their harmful effects, regulating their use. Several studies support a positive association between long-term exposure to pesticides and chronic pathologies, such as cancer. Geno-toxicological biomonitoring has proven to be valuable to assess genetic risks associated with exposure to pesticides, representing a promising tool to improve preventive measures and identify workers at higher risk. In this study, a differential gene expression analysis of 70 candidate genes deregulated upon pesticide exposure, was performed in 10 GEO human gene expression DataSets. It was found that six genes (PMAIP1, GCLM, CD36, SQSTM1, ABCC3, NR4A2) had significant AUC predictive values. Also, CD36 was upregulated in non-transformed cell samples and healthy workers, but downregulated in cancer cells. Further validation in larger groups of workers will corroborate the importance of the identified candidates as biomarkers of exposure/effect.

1. Introduction

Pesticides, including insecticides, fungicides, herbicides, plant growth regulators, rodenticides, are pivotal compounds for our global economy, given their valuable efficacy in controlling pests and reducing plant diseases. During the latest 25 years, worldwide pesticide production and use has increased by 30 %, reaching up to 2.7 million tons by 2020, with the largest contributions to pesticide use coming from US, Asia, Europe, Africa, and Oceania (World Food and Agriculture, 2022).

Despite their benefits, pesticides are generally considered hazardous substances for living organisms, including humans, due to their chemical properties, potential for bioaccumulation, and limited selectivity (Gattuso et al., 2022; Schwingl et al., 2021). Several studies demonstrated that exposure to pesticides, in both environmental and occupational settings, may represent a substantial risk factor for human health, being associated with neurodegenerative, immunological, and hormonal disorders (Fenga et al., 2017; Li et al., 2019; Navarrete-Meneses and Pérez-Vera, 2019; Sabarwal et al., 2018; Syeda and Cannon, 2021).

Importantly, a growing number of pesticides have been classified as known or suspected human carcinogens by authoritative organizations, such as the International Agency for Research on Cancer (IARC) (Guha et al., 2016). Hence, many of them are currently banned or subjected to restricted usage within the EU (European Parliament 2009, 2005). Generally, regulations are less stringent outside EU, especially in developing countries (including Brazil, Mexico, Indonesia, Malaysia, Colombia, Ukraine, Peru, South Africa, the Russian Federation, and Morocco), where their employment in agriculture is often scarcely controlled thus increasing health hazard potentially linked with occupational exposure (Sarkar et al., 2021).

At the cellular level, pathogenic mechanisms underneath pesticide toxicity involve both genetic and epigenetic alterations that deeply impact the function of crucial genes and enzymes participating in several molecular processes, and potentially triggering the development of either neoplastic or neurodegenerative disorders (Giambò et al., 2021). Several pesticide-associated genetic alterations are currently known (Teodoro et al., 2019); mutagenic mechanisms impacting

Abbreviations: GEO, Gene Expression Omnibus; RNA-seq, RNA Sequencing; ROC, Receiver Operating Characteristic curve; ROS, Reactive Oxygen Species.

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oncogenes have been extensively investigated *in vitro*, and there is also human evidence linking pesticide exposure to mutations in critical genes associated with cancer initiation and progression (Vakonaki et al., 2013). In general, DNA damage, oxidative stress, with concurrent alteration of gene expression, DNA methylation and miRNA levels, have been observed in hematopoietic tissues upon exposure to insecticides or fungicides (Navarrete-Meneses et al., 2023).

Given the large variety of active substances that may be component of pesticides, assessing the risk associated with pesticide exposure represents a current challenge. Estimating exposure is crucial to differentiate risk levels among individuals, as well as to distinguish between low, medium, and high levels of exposure. However, exposure assessment, typically defined through observational studies using indexes and scales, requires validation through assessment of biological responses, such as by measuring specific biomarkers (Filippi et al., 2021). There is an actual need for novel appropriate biomarkers to facilitate early detection of adverse effects and sub-clinical damage potentially linked with occupational exposure to pesticides.

Biomarker research has gained significant interest in the recent years due to its potential in supporting both prevention and intervention strategies to mitigate health risks linked to uncontrolled exposure to these compounds (Lionetto et al., 2019). For example, organophosphates and carbamates are known to induce toxicity by inhibiting acetylcholinesterase. Conversely, suitable effect biomarkers for other pesticides are lacking (Gupta et al., 2018). Thus far, geno-toxicological biomonitoring has proven to be very valuable for assessing genetic risks arising from exposure to multiple chemicals (Bolognesi, 2003). Hence, analyzing gene expression alterations in individuals chronically exposed to these harmful chemicals holds a great potential for early-predicting the emergence of pathological conditions directly linked with the exposure, including cancer (Cavalier et al., 2023).

On these bases, the primary objective of this study was to identify a panel of genes whose expression is potentially altered in association with chronic exposure to pesticides. To achieve this goal, the expression of a selected panel of 70 genes, found altered upon pesticide exposure in previous screening-based studies, has been analysed in a total of ten selected Gene Expression Omnibus (GEO) DataSets. Specifically, they are deposited expression datasets of cells and workers exposed (or not) to the main categories of pesticides. Downstream pathways potentially activated by these genes, as well as miRNAs potentially involved in upstream post-transcriptional gene regulation have been thoroughly investigated. Once identified, these genes might represent valuable indicators of both early exposure and sub-lethal damage. Additionally, they might help to stratify workers based on their individual susceptibility. Finally, they may represent indicators of detrimental health effects possibly linked with pesticide use, such as cancer.

2. Materials and methods

2.1. Literature search

A literature search was performed on PubMed repository to identify full-text articles published in the last 5 years using the following keywords: ("pesticidal"[All Fields] OR "pesticide s"[All Fields] OR "pesticides"[Pharmacological Action] OR "pesticides"[MeSH Terms] OR "pesticides"[All Fields] OR "pesticide"[All Fields]) AND ("gene expression"[MeSH Terms] OR ("gene"[All Fields] AND "expression"[All Fields]) OR "gene expression"[All Fields]). The search was conducted on January 15th, 2023 and it produced a total of 495 results. Later refinement through manual filtering allowed the exclusion of a total of 324 articles. Overall, a total of 170 original articles were selected for further analysis.

Among the 170 shortlisted original articles, 15 studies were further selected, on the basis that they specifically employed either microarray or RNA-sequencing as measurements of gene expression (Anjitha et al., 2020; Harris et al., 2018; Huang et al., 2019; Jerber et al., 2021; Knebel

et al., 2022; Lauschke et al., 2021; Liu et al., 2022; Lu et al., 2022; Lv et al., 2021; Pamies et al., 2018; Seeger et al., 2019; Stur et al., 2019; Tombuloglu et al., 2020; Tong et al., 2020; Wang et al., 2021). A flow chart of this process is presented in Fig. 1.

2.2. Selection of candidate genetic biomarkers and functional analyses

The Gene Expression Omnibus (GEO) DataSets database, available on NCBI (www.ncbi.nlm.nih.gov/geo/, last accessed on 26 April 2023), has been used to select the gene expression datasets of human cells or workers exposed to pesticides. In particular, an advanced search using the following search terms was performed: "(pesticides AND 'Homo sapiens' [porgn:txid9606])". Deposited datasets containing expression data on human samples or human cell lines, which were exposed to pesticides, have been included. Datasets deriving from toxicity studies performed in animals or vegetables, or composed of less than 10 samples were excluded. A total of ten datasets were shortlisted for further computational analysis, as reported in Table 1.

Following the download of gene expression data matrices from GEO DataSets, differential analyses were conducted between exposed and non-exposed samples, in order to detect genes whose expression was deregulated following exposure to pesticides. To this end, the GEO2R bioinformatic tool, accessible on the GEO DataSets database, was used. In detail, samples were clustered into "exposed samples group" and "non-exposed control group", to further perform the differential analysis of selected gene expression (i.e., genes identified through literature-available results) (Barrett et al., 2012).

Furthermore, in order to better understand the role of the deregulated genes, an enrichment analysis was conducted through g:Profiler online tool (Kolberg et al., 2023a). In particular, the function g:GOST was used to perform functional enrichment analysis, referred to as over-representation analysis or gene set enrichment analysis, using input gene lists. This process involved the association of genes with

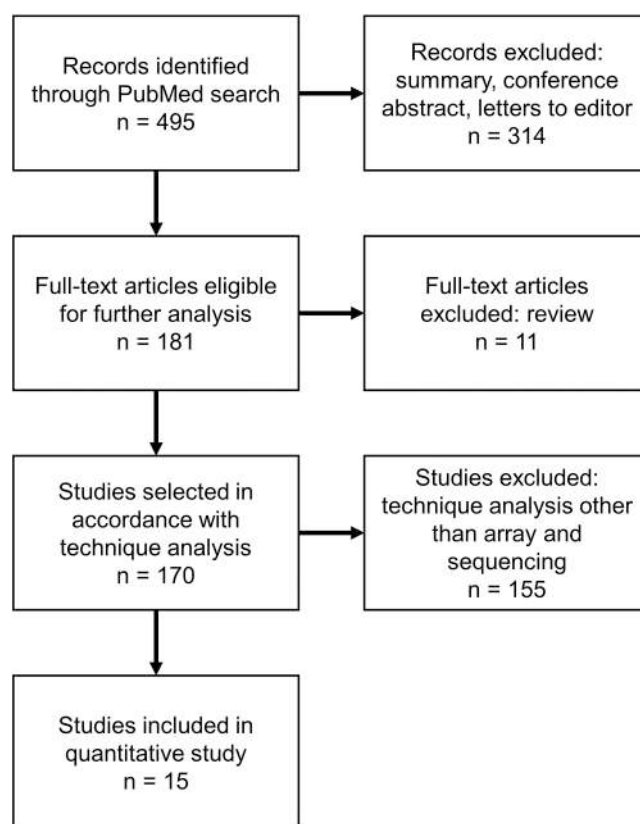


Fig. 1. Flow chart of literature search.

Table 1

Features of the GEO DataSets used in the study.

Dataset ID	Platform	Sample Type (human)	Exposed Samples (N)	Control Samples (N)	Ref
GSE30335	GPL570 Affymetrix HG U133 Plus 2.0 Array	Peripheral blood cells	Farm workers (20)	Manual workers (20)	(Jiang et al., 2022)
GSE94521	GPL570 Affymetrix HG U133 Plus 2.0 Array	Human embryonic stem cells derived from neural crest cells (hESC-NCC)	Arsenic trioxide (5) Cyproconazole (5) Triadimefon (5)	Control (5) Control (5)	(Pallocca et al., 2017)
GSE109565	GPL18573 Illumina NextSeq 500 <i>Homo sapiens</i>	Liver normal cells (HepaRG)	Glyphosate (15) Roundup (5)	Control (5)	(Mesnage et al., 2018a)
GSE109511	GPL13158 Affymetrix HT HG-U133 Plus PM Array	Liver normal cells (HepaRG)	Fenbuconazole (27) Imazalil (27) 2,4-dichloro-phenoxyacetic acid (27)	Control (3) Control (3) Control (3)	(Haider et al., 2018)
GSE114573	GPL18573 Illumina NextSeq 500 <i>Homo sapiens</i>	Liver normal cells (HepaRG)	Quisqualop-p-ethyl (15) Isoxaflutole (15) Mesotrione (15)	Control (5)	(Mesnage et al., 2018b)
GSE35642	GPL96 Affymetrix HG U133A Array	Neuroblastoma cells (SK-N-MC)	Rotenone (12)	Control (6)	(Cabeza-Arvelaiz and Schiestl, 2012)
GSE4773	GPL570 Affymetrix HG U133 Plus 2.0 Array	Neuroblastoma cells (SK-N-MC)	Rotenone (12)	Control (12)	NA
GSE109513	GPL13158 Affymetrix HT HG-U133 Plus PM Array	Hepatocellular carcinoma cells (HepG2)	Fenbuconazole (27) Imazalil (27) 2,4-dichloro-phenoxyacetic acid (27)	Control (3) Control (3) Control (3)	(Haider et al., 2018)
GSE109508	GPL13158 Affymetrix HT HG-U133 Plus PM Array	Rhabdomyosarcoma cells (A673)	Fenbuconazole (27) Imazalil (27) 2,4-dichloro-phenoxyacetic acid (27)	Control (3) Control (3) Control (3)	(Haider et al., 2018)
GSE109509	GPL13158 Affymetrix HT HG-U133 Plus PM Array	Breast adenocarcinoma cells (MCF7)	Fenbuconazole (27) Imazalil (27) 2,4-dichloro-phenoxyacetic acid (27)	Control (3) Control (3) Control (3)	(Haider et al., 2018)

NA= not associated.

established functional information sources to characterize statistically significant enrichment of terms (Kolberg et al., 2023b). Additionally, STRING v11.0 bioinformatic tool was used to analyse interaction networks of differentially expressed genes (Szklarczyk et al., 2021).

To identify miRNAs regulating the expression of genes whose expression was found altered in GEO DataSets upon pesticide exposure, miRNA regulatory network analysis was performed by using MirNet online tool (which integrates data from 14 different miRNA databases TarBase, miRTarBase, miRecords, miRanda, miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, starBase, TransmiR, ADmiRE, and TAM 2.0) (Chang et al., 2020). Upon uploading specific gene lists, online available filters were selected to further refine the analysis (i.e., degree cutoff, betweenness centrality, shortest path filter, minimum network).

2.3. Statistical analysis

Fold change values obtained through the differential expression analyses performed by using GEO2R software were expressed as log₂FC. Data processing and statistical analyses were performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, La Jolla, CA, USA). The results were presented as average $\pm$ standard deviation (SD). For each variable of interest, Shapiro–Wilk normality tests were run. For normally distributed data, single-parameter comparisons between two groups were conducted using two-tailed unpaired Student's t-tests. For non-normally distributed data, non-parametric tests were used, and single-parameter comparisons between two groups were conducted using Mann–Whitney tests. To assess the prognostic significance of deregulated genes, receiver operating characteristic curve (ROC) analyses were performed, and the corresponding areas under the curve (AUC) were calculated. Specifically, for each individual gene analysed, samples were stratified in two classes (exposed vs non-exposed) and

subsequently the two groups were analysed through the ROC function analysis of Prism software. For all statistical analyses, differences were considered significant with p-values < 0.05; with * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

3. Results

3.1. Literature data analysis allowed the identification of 70 genes with altered expression upon pesticide exposure

Following the literature search (previous 5 years) and results analyses, a total of 15 research articles were shortlisted because they satisfied the pre-established inclusion and exclusion criteria. The included studies were conducted in cells exposed or not, to different pesticides, which was then followed by gene expression analysis using either microarray or RNA sequencing readout techniques. The list of the 70 genes whose expression was found significantly affected by pesticide exposure is summarized in Table 2.

The set of 70 genes encode proteins that are involved in several biological processes, including homeostasis, cellular response to chemical stimuli, regulation of metabolism, lipids adsorption and catabolism (Supplementary Fig.1), and the majority of the protein products of the 70 genes are functionally interconnected (Supplementary Fig.2).

3.2. Identification of potential biomarkers of exposure in normal cells treated with pesticides

The differential analyses were performed on four different gene expression datasets, with either neuronal cells (hESC-NCC, 1 dataset) or hepatocytes (HepaRG, 3 datasets), treated versus untreated with 11 diverse pesticides (Fig. 2). These analyses allowed the identification of differentially expressed genes for each of the datasets. By merging these

Table 2

List of 70 genes selected from data literature analysis.

Gene Name	Gene ID	Regulation after treatment	Pesticide Class	Active substance(s)	Sample Type (human)	Method	Reference
ABCB11	8647	Down	Fungicide	Propiconazole Tebuconazole	Liver normal cells (HepaRG)	<i>Microarray</i> HGE 8x60K Agilent Technology	(Knebel et al., 2022)
ABCC3	8714	Up					
ABCG5	64240	Up					
CYP7A1	1581	Down					
CYP8B1	1582	Down					
CYP1A1	1543	Up	Fungicide	Cyproconazole Epoxiconazole Prochloraz	Liver normal cells (HepaRG)	<i>Microarray</i> HE G3 8x60Kv2 Agilent Technology	(Seeger et al., 2019)
LSS	4047	Up					
TM7SF2	7108	Up					
ATP1A2	477	Down					
CLDN16	10686	Down					
KRTAP2-2	728279	Down	Fungicide	Mancozeb	Endometrial adenocarcinoma cells (Ishikawa) Choriocarcinoma cells (JEG-3)	<i>Microarray</i> Affymetrix HTA 2.0 ST Thermo Fisher Scientific	(Wang et al., 2021)
GCLC	2729	Up					
GCLM	2730	Up					
HMOX1	3162	Up					
NCF2	4688	Up					
SLC6A6	6533	Up	Insecticide	Diallyl disulfide (DADS) Diallyl trisulfide (DATS) Diallyl tetrasulfide (DTS)	Hepatocellular carcinoma cells (HepG2)	<i>Microarray</i> Affymetrix HG U133 Plus 2.0 Thermo Fisher Scientific	(Lv et al., 2021)
SQSTM1	8878	Up					
ACOX2	8309	Up					
MTHFD1	4522	Up					
BNIP3	664	Down					
FAM162A	26335	Down	Herbicide	Atrazine	Breast adenocarcinoma cells (MCF7)	<i>RNA-seq</i> HiSeq Illumina	(Lu et al., 2022)
PGK1	5230	Down					
GDF3	9573	Up					
GFAP	2670	Up					
NGN1	177045	Down					
OCT4	5460	Up	Herbicide	Glyphosate Roundup Aminomethyl-phosphonic acid (AMPA)	Breast adenocarcinoma cells (MCF7, MDA-MB-468)	<i>Microarray</i> Affymetrix HTA 2.0 ST Thermo Fisher Scientific	(Stur et al., 2019)
PAX6	5080	Down					
S100B	6285	Up					
SOX1	6656	Down					
AKT2	208	Down					
AKT3	10000	Down	Insecticide	Paraquat	Human umbilical cord blood-neural stem cells (HUCB-NSCs)	<i>RNA-seq</i> HiSeq2500 Illumina	(Huang et al., 2019)
ATP6V0D2	245972	Up					
CAB39L	81617	Up					
CCND2	894	Up					
CD36	948	Up					
EGF	1950	Down	Insecticide	Nitenpyram	Human bone marrow mesenchymal stem cells (hBMSCs)	<i>Microarray</i> Affymetrix PrimeView HGE Thermo Fisher Scientific	(Liu et al., 2022)
EIF4B	1975	Down					
FGF2	2247	Up					
MDM2	4193	Up					
PDGFRB	5159	Down					
PPP2R2C	5522	Up	Insecticide	Malathion	Peripheral blood cells	<i>Microarray</i> HGE 8x60Kv2 Agilent Technology	(Anjitha et al., 2020)
RAGD	58528	Up					
THBS1	7057	Down					
BM1I	648	Up					
HLA-E	3133	Down					
LPCAT4	254531	Up	Insecticide	Rotenone	Brain cells	<i>Microarray</i> HGE 8x60Kv2 Agilent Technology	(Jerber et al., 2021)
MAP2K3	5606	Down					
MICA	100507436	Up					
ACSF3	197322	NA					
SFXN5	94097	NA					
ATF4	468	Down	Insecticide	Rotenone	Neuronal-like cells (LUHMES)	<i>Microarray</i> HE G3 8x60Kv2 Agilent Technology	(Harris et al., 2018)
CASP3	836	Up					
DAT	6531	Down					
EAAC1	6505	Down					
MLF1IP	79682	Down					
NEF2L2	4780	Down	Insecticide	Rotenone	Human induced pluripotent stem cells (iPSC) from fibroblasts	<i>Microarray</i> HE G3 8x60Kv2 Agilent Technology	(Pamies et al., 2018)
TYMS	7298	Down					
ATP6V1G2	534	Down					
DCX	1641	Down					
FGF14	2259	Down					
NR4A2	4929	Down	Insecticide	Boric acid Borax	Hepatocellular carcinoma cells (HepG2)	<i>Microarray</i> Affymetrix HTA 1.0 ST	(Tombuloglu et al., 2020)
EI24	9538	NA					
FAS	355	NA					
GADD45	35646	NA					
P21	1026	NA					

(continued on next page)

Table 2 (continued)

Gene Name	Gene ID	Regulation after treatment	Pesticide Class	Active substance(s)	Sample Type (human)	Method	Reference
PMAIP1	5366	NA	Insecticide and Fungicide	Rotenone Ziram	Neuronal-like cells (LUHMES)	Thermo Fisher Scientific RNA-seq HiSeq2000 Illumina	(Tong et al., 2020)
TP53I3	9540	NA					
DDIT3	1649	Up					
MT1G	4495	Up					
PDK4	5166	Up					

NA= not associated.

DATASET Compound	GSE94521 Arsenic trioxide	GSE94521 Cyproconazole	GSE94521 Triadimefon	GSE109565 Glyphosate	GSE109565 Roundup	GSE109511 2,4-D	GSE109511 Fenbuconazole	GSE109511 Imazalil	GSE114573 Isoxaflutole	GSE114573 Mesotrione	GSE114573 Quizalofop
Gene ID	hESC-NCC			HepaRG							
GCLM	1,378					0,456	0,496	0,521	0,254		1,245
AKT3	0,377	-0,380				0,349	0,384	0,335			0,334
FAS	0,481	0,246	-0,355			0,219	0,277	0,310			
CYP1A1		-0,245			-2,227				2,102	1,320	5,701
CD36		0,318				0,226	0,204				1,181
PMAIP1		0,262	0,486							0,290	1,276
EIF4B	0,250	0,216	0,330				0,174				
FGF14	-0,933	0,286				0,286				-0,565	
GCLC	0,283	-0,265							0,136		0,722
AKT2	-0,384		0,341				0,321				
SLC6A6	-0,477		0,480		-0,151						
NR4A2	-0,950	-0,576	-0,473								
PGK1	0,327	0,431	-0,175								
EGF	0,759	0,233		-0,215							
THBS1	0,385		-0,822				0,440				
HMOX1	4,959		-0,321								1,216
ATF4	0,251								-0,120		1,234
DCX	-0,479		0,318			-0,169					
FAM162A	0,361		0,228					-0,254			
SQSTM1	0,811		0,159								1,188
PDK4	-0,230								0,690		2,425
MDM2	1,342		-0,157								
SOX1	-1,274	0,272									
LSS	-0,201	0,257									
CAB39L	0,453		-0,450								
LPCAT4	0,693	0,358									
ABCB11		-0,274	-0,304								
CCND2	0,414	0,692									
ABCC3		-0,376	-0,303								
S100B	-0,395							-0,177			
FGF2	0,467		-0,390								
BMI1									0,185		0,172
TM7SF2		0,511	0,380								
PDGFRB	-0,332										0,677
PPP2R2C			0,437								
GFAP	0,236										
MT1G	1,199										
BNIP3	0,196										
HLA-E	0,335										
TP53I3	0,193										
ACSF3		-0,270									
CYP7A1					0,491						
MICA											0,475
MTHFD1	-0,343										
NCF2											1,208
PAX6		-0,480									

Fig. 2. Differentially expressed genes after pesticide exposure in normal cell samples. The fold change values are expressed as log₂ of fold change. Upregulated genes are highlighted with red scale while downregulated genes are highlighted with blue scale. Highlighted in red are the gene IDs corresponding to the 13 genes concurrently altered upon more than one pesticide treatment.

lists, it was possible to identify a set of 46 genes significantly upregulated or downregulated as a consequence of pesticide exposure in at least one treatment. Out of these 46 genes, 13 were concurrently upregulated (GCLM, CD36, PMAIP1, EIF4B, SQSTM1, LPCAT4, CCND2, BMI1, TM7SF2) or downregulated (NR4A2, ABCB11, ABCC3, S100B) upon at least two different treatments, and these were further explored as potential biomarker of exposure to pesticides.

Furthermore, a functional enrichment analysis was conducted on the list of 13 significantly deregulated genes, by using the g:GOST tool of g:profiler online analysis software. Regarding the gene ontology (GO) terms, 12 molecular functions (MF) were found significantly affected specifically by ABCB11, ABCC3, and CD36. In particular, 3 of these functions contained driver terms in GO (i.e., ATPase-couples organic acid transmembrane transporter activity, monocarboxylic acid transmembrane transporter activity and ABC-type xenobiotic transporter activity). Additionally, 1 biological process (BP) is significantly affected,

that is the response to oxidative stress, in which 5 of the deregulated genes are involved (GCLM, CD36, NR4A2, SQSTM1, ABCB11). From pathways enrichment analysis, 6 WikiPathways (WP) are significantly affected, including cholesterol metabolism, bile acid enterohepatic circulation, drug induction and bile acids pathway, cholestasis, IL-24 signalling and photodynamic therapy survival signalling, with 7 genes differentially involved (GCLM, CD36, PMAIP1, SQSTM1, ABCB11, ABCC3, BMI1, TM7SF2). The results are summarized in Fig. 3.

Finally, in order to identify which miRNAs were involved in the post-transcriptional regulation of the expression of the 13 deregulated genes in normal cells, a miRNet analysis was performed. The regulatory network shown in Fig. 4 highlights a total of 9 miRNAs, 7 of which modulate the expression of two genes (hsa-mir-1343-5p, hsa-mir-182-5p, hsa-mir-522-5p, hsa-mir-191-5p, hsa-mir-23b-3p, hsa-mir-429, hsa-mir-27a-5p) and 2 of which modulate the expression of 3 genes (hsa-mir-7-5p, hsa-mir-26b-5p).



Fig. 3. g:GOST functional network analysis of differentially expressed genes after pesticide exposure in normal cell samples. **A.** g:GOST multiquery Manhattan plot, X-axis shows the functional terms grouped and color-coded by data source, Y-axis shows the $-\log_{10}$ of P adjusted values. Highlighted drivers GO terms are numbered from 1 to 4. **B.** Table summarizing multiquery results with significant P adjusted values and genes involved. MF, Molecular Function; BP, Biological Process, WP, Wiki Pathway. Highlighted in blue: diver GO terms.

3.3. Identification of potential biomarkers of exposure in cancer cells treated with pesticides

The differential analyses were performed on five different gene expression datasets, and carried out with different cancer cell lines. Specifically, neuroblastoma (SK-N-MC), hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7), or rhabdomyosarcoma (A673) were employed in the experiments, either treated or untreated with 4 different pesticides (Fig. 5). These analyses allowed the identification of differentially expressed genes for each of the datasets. By merging these lists, it was possible to identify a set of 30 genes significantly upregulated or downregulated as a consequence of pesticide exposure in at least one treatment. Out of these genes, 12 were concurrently upregulated (MDM2, LSS, EGF, TYMS) or downregulated (SOX1, PPP2R2C, CD36, EIF4B, FGF14, THBS1, CAB39L, CYP8B1) following at least two different treatments. Overall, they can be explored as potential biomarkers of transformation whose expression is affected by pesticide exposure.

Additionally, a functional enrichment analysis was conducted on the list of 12 significantly deregulated genes, by using the g:GOST tool of g: profiler online analysis software. Regarding the gene ontology (GO) terms, 7 MF have been found significantly affected specifically by CD36, EGF, THBS1, CAB39L. In particular, 3 of these functions contained driver terms in GO (i.e., low-density lipoprotein particle binding, protein kinase activator activity, TGF- β binding). Additionally, 6 BP are significantly affected, and 3 of them contain driver GO terms (regulation of lipid localization, long chain fatty acid import, cholesterol transport). In these BP a total of 4 genes are variously involved: CD36, EGF, THBS1, CYP8B1. Finally, 2 GO cellular components (CC), which are also driver terms in GO are affected (plasma alpha granule and endocytic vesicle membrane), by a subset of 4 of the 12 genes (MDM2, CD36, EGF, THBS1). From pathways enrichment analysis, 4 KEGG pathways are significantly affected by a differential combination of 7 of the 12 genes (MDM2, PPP2R2C, CD36, EIF4B, EGF, THBS1, CAB39L). In particular, WP analysis confirms the involvement of PI3K-Akt signalling pathway found among the ones identified with KEGG and also in this case the

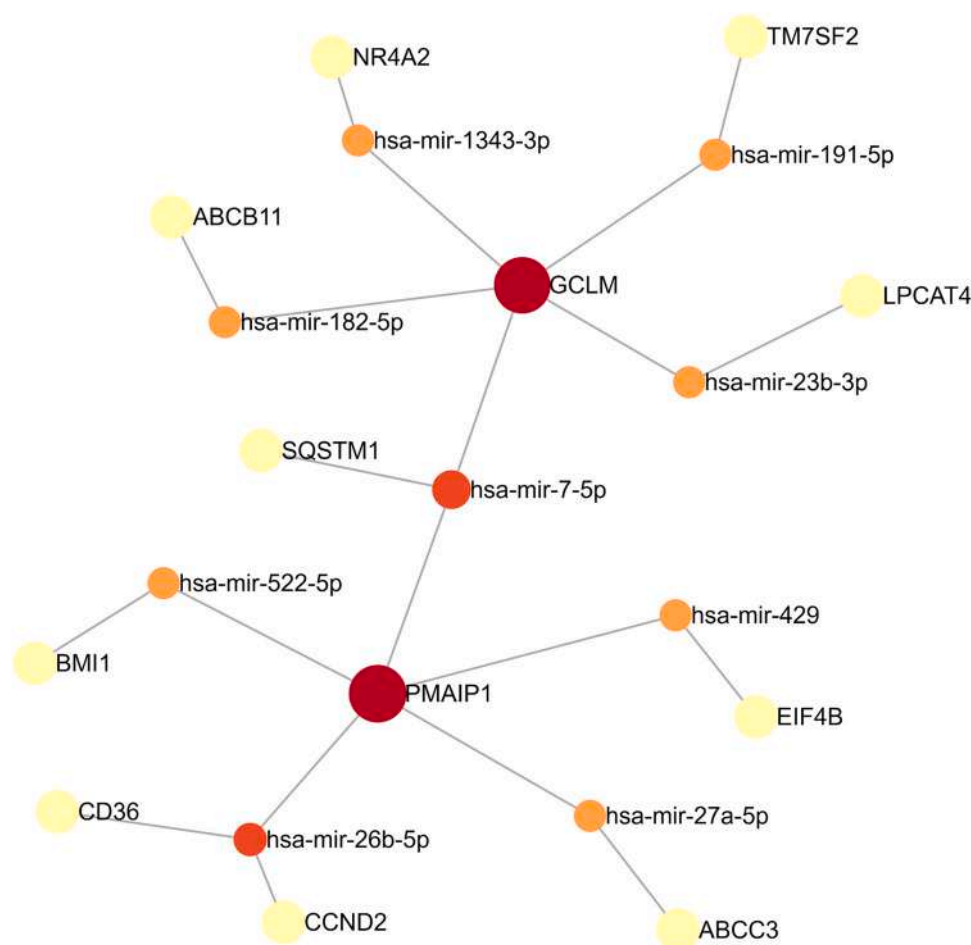


Fig. 4. miRNet miRNA-target interaction network of 13 differentially expressed genes and 9 miRNAs after pesticide exposure in normal cell samples.

same 7 genes are involved (MDM2, PPP2R2C, CD36, EIF4B, EGF, THBS1, CAB39L). Finally, within the reactome the platelet degranulation is confirmed as found in GO:CC, always involving CD36, EGF and THBS1. The results are summarized below in Fig. 6.

Furthermore, in order to identify which miRNAs are involved in the post-transcriptional regulation of the 12 deregulated genes in cancer cells, a miRNet analysis was performed. The regulatory network shown in Fig. 7 highlights a total of 8 miRNAs, 5 of which modulate the expression of two genes (hsa-mir-665, hsa-mir-194-5p, hsa-mir-30a-5p, hsa-mir-26b-5p, hsa-let-7a-5p) and 3 of which modulate the expression of 3 genes (hsa-mir-182-5p, hsa-mir-26a-5p, hsa-mir-218-5p).

3.4. Identification of biomarkers of cellular neoplastic transformation linked with pesticide exposure in normal versus cancer cells

Results from differential analysis of normal and tumor cell datasets were compared to search for gene changes present in both identified sets. As shown below in Fig. 8A, two genes are differentially expressed in both normal and cancer datasets: CD36 and EIF4B. Importantly, while both genes are upregulated after pesticide treatments in normal cells, they are downregulated after treatments in tumour cellular samples.

Moreover, through miRNet analysis, it is possible to identify miRNAs involved in the regulation of both CD36 and EIF4B levels (i.e., hsa-mir-129-2-3p, hsa-mir-124-3p, hsa-mir-155-5p, hsa-mir-16-5p). Subsequent miRNA-disease analysis evidenced that 15 out of 21 diseases are cancer-related, corresponding to the 72 % of the total (Fig. 8B). This result suggests that CD36 and EIF4B may be proposed as biomarkers of pesticide related-toxicity in both normal and cancer cells, with opposite outcomes in terms of effect on their level of expression. Moreover, hsa-

mir-129-2-3p, hsa-mir-124-3p, hsa-mir-155-5p, hsa-mir-16-5p may be further explored as epigenetic biomarkers possibly modulating cell-type dependent post-transcriptional modulation of such genes.

3.5. Validation of biomarkers of exposure and effect in farm workers chronically exposed to pesticides

There was a further investigation of the relevance of the genes identified in the datasets of cells (normal and cancerous) and their possible use as biomarkers to assess pesticide exposure. To this aim, a differential gene expression analysis was performed in whole blood cell samples from 20 male Latino farm workers chronically exposed to agricultural pesticides and herbicides, and 20 age-matched male manual workers as non-exposed controls (GEO DataSet GSE30335).

The analysis allowed the identification of differentially expressed genes within the dataset. In particular, out of 70 genes analysed, it was possible to identify a set of 22 genes that were significantly upregulated (PMAIP1, MDM2, GCLM, CD36, PGK1, TP53I3, SQSTM1, FAS, CAB39L) or downregulated (FGF2, PPP2R2C, DCX, GFAP, TM7SF2, SOX1, MT1G, PDGFRB, ABCC3, LSS, TYMS, NR4A2, THBS1) as a consequence of prolonged pesticide exposure in farm workers when compared to their expression in manual workers (Fig. 9A).

Furthermore, the functional enrichment analysis conducted on the list of 22 significantly deregulated genes, by using the g:GOST tool of g: profiler online analysis software, evidenced several pathways functionally enriched. Regarding the gene ontology (GO) terms, 1 MF that is also a driver term in GO, the low-density lipoprotein particle binding (specifically by CD36 and THBS1), was found significantly affected. Additionally, 27 BP were significantly affected, 7 of which contain

DATASET	GSE4773	GSE35462	GSE109513	GSE109509	GSE109508	GSE109513	GSE109509	GSE109508	GSE109513	GSE109509
Cell line	SK-N-MC	SK-N-MC	HepG2	MCF7	A673	HepG2	MCF7	A673	HepG2	MCF7
Gene ID	Rotenone		Fenbuconazole		Imazalil		2,4-D			
MDM2	0,653			0,186	0,368		0,239	0,147		0,235
AKT2	-1,290	-0,609			0,424		0,333			
SLC6A6		-0,615			0,180		0,288			0,189
SGX1				-0,213	-0,298		-0,185			
LSS			0,306				0,411			0,418
PPP2R2C					-0,349			-0,208	-0,387	
GCLM	0,261			-0,159	-0,187					-0,155
CD36		0,537						-0,224		
NR4A2		-0,275								
EIF4B	-0,275	-0,405								
FGF14	-0,962				-0,210					
PGK1	0,299	-0,358								
EGF	0,325		0,152							
THBS1	-0,915							-0,240		
CAB39L	-1,290					-0,249				
LPCAT4		0,459						-0,238		
GFAP	-0,640	0,392								
CYP8B1	-0,532			-0,161						
TYMS	0,595							0,319		
AKT3								-0,196		
FAS					0,312					
CYP1A1		0,582								
PMAIP1	0,545									
HMOX1		-0,736								
ABCB11		0,521								
CCND2		-0,377								
MT1G						0,207				
S100B	0,752									
BNIP3		-0,579								
HLA-E	-0,513									

Fig. 5. Differentially expressed genes after pesticide exposure in cancer cell samples. The fold change values are expressed as $\log_2$ of fold change. Upregulated genes are highlighted with red scale while downregulated genes are highlighted with blue scale. Highlighted in red the 12 gene IDs concurrently altered upon more than one pesticide treatment.

driver GO terms (cellular response to chemical stimuli, regulation of smooth muscle cell growth, regulation of apoptotic pathway, positive regulation of protein phosphorylation and catalytic activity, response to extracellular stimuli, angiogenesis in cardiovascular morphogenesis cellular response to external stimuli, response to endogenous stimuli). In particular, in each identified BP, 18 out of 22 genes are variously involved. From pathways enrichment analysis, 3 KEGG pathways (p53 signalling, platinum drug resistance and melanoma) are significantly affected by a differential combination of 8 genes (FAS, PMAIP1, THBS1, MDM2, FGF2, PDGFRB, TP53I3). In particular, WP analysis confirms the involvement of p53 signalling pathway found among the ones identified with KEGG and 12 genes are involved (FAS, CD36, PMAIP1, THBS1, MDM2, LSS, CAB39L, FGF2, TM7FS2, PDGFRB, PPPR2C, TP53I3). Finally, also within the REAC the involvement of TP53 mediated regulation of transcription of cell death genes was confirmed (for FAS, PMAIP1 and TP53I3; Fig. 9B and C).

Finally, in order to identify which miRNAs are involved in the upstream regulation of the expression of the 22 deregulated genes in farm workers chronically exposed to pesticides, a miRNet analysis was performed. The regulatory network shown in Fig. 10 highlights a total of 10 miRNAs. Four of them (hsa-mir-21-5p, hsa-mir-218-5p, hsa-mir-200b-3p, hsa-mir-194-5p) synergistically regulate the post transcriptional expression of 4 genes, whereas 3 of them (hsa-mir-192-5p, hsa-let-7a-5p, hsa-mir-24-3p) modulate synergistically 3 genes, and 3 (hsa-mir-26b-5p, hsa-mir-27a-3p, hsa-mir-26a-5p) modulate synergistically 2 genes.

Importantly, the outcomes of differential gene expression analyses conducted within the farm worker dataset, was crossed with differentially expressed genes within all the DataSets of normal cells exposed to pesticides. From the intersection of the outcomes, a total of 6 genes are mutually deregulated (Fig. 11A). While PMAIP1, GCLM, CD36 and SQSTM1 are upregulated in farm workers (FW) compared with manual workers (MW), on the contrary ABCC3 and NR4A2 are downregulated. Consistently, the ROC curves showed all significant AUC values. These high and significant AUC performances suggested that all 6 differentially

expressed genes can be considered as diagnostic discriminators between workers exposed or non-exposed to pesticides for a prolonged period of time. The differential expression of such genes and the ROC analyses are reported in Fig. 11B.

Moreover, the STRING network analysis highlighted that protein products corresponding to 3 genes out of 6 (i.e. GCLM, SQSTM1 and PMAIP1) are functionally interconnected, suggesting their coordinated response to pesticide detrimental exposure in healthy subjects (Fig. 11C).

Finally, by comparing genes deregulated in farm workers exposed to pesticides with set of genes obtained from the differential analyses of both normal and transformed cellular sample DataSets, one gene, CD36, is concurrently altered. In particular, while in normal cells is upregulated following pesticide treatment as it was in farm workers, on the contrary in cancer cells CD36 is downregulated following pesticide treatment. These data suggest a dual role for CD36 gene, both as susceptibility and effect biological indicator (Fig. 12).

4. Discussion

Pesticides are widely utilized products, from inside our houses to outside in the countryside. Several situations can be associated with pesticide exposure, either occupational or not, such as pesticide synthesis, transportation, use in the fields, selling and consuming agricultural food. The level of exposure might vary, being short-term and high-level, in case of intentional or occupational-accidental. Also, the exposure can be long-term high-level (frequently occupational), or low-level (typically non-occupational). Regarding the occupational exposure, it accounts for about the 40 % of the total risk (Sabarwal et al., 2018). Subjects exposed to pesticides, including those that work in close contact for long hours with such substances, were found to be at greater risk to develop different cancers including leukaemia, lymphoma, neuroblastoma, sarcoma of soft tissues, cancers of lung, stomach, colon, rectum, bladder, ovaries, and prostate (Bonner et al., 2017; Dich et al., 1997; Hu et al., 2017; Lerro et al., 2015; Luo et al., 2016; Parrón et al., 2014;



Fig. 6. g:GOST functional network analysis of differentially expressed genes after pesticide exposure in cancer cell samples. **A.** g:GOST multiquery Manhattan plot, X-axis shows the functional terms grouped and color-coded by data source, Y-axis shows the $-\log_{10}$ of P adjusted values. Highlighted drivers GO terms are numbered from 1 to 8. **B.** Table summarizing multiquery results with significant P adjusted values and genes involved. MF, Molecular Function; BP, Biological Process, CC, Cellular Component, KEGG, Kyoto Encyclopedia of Genes and Genomes, REAC, Reactome, WP, Wiki Pathway. Highlighted in blue: diver GO terms.

Polanco Rodríguez et al., 2017; Schinasi and Leon, 2014; Xu et al., 2010). Also, many other pathologies are variously associated with pesticide exposure including Parkinson's disease, Alzheimer's disease, reprotoxicity, allergy and respiratory disorders (Fenga et al., 2021; Vellingiri et al., 2022; Zaganas et al., 2013; Zhao et al., 2023).

Toxic effects of pesticides are largely studied, through observational studies involving workers exposed to such substances, as well as through

preclinical ones (Lushchak et al., 2018). Pesticides, like other xenobiotics, often trigger defensive biotransformational responses: these activate nuclear and cytosolic molecular sensors, which upregulate the expression of biotransforming enzymes and transporter systems aimed at eliminating them from the organism. These molecules are disruptors of the endocrine functions, especially those driven by thyroid and reproductive hormones (Zhang et al., 2020). As a common feature, is the

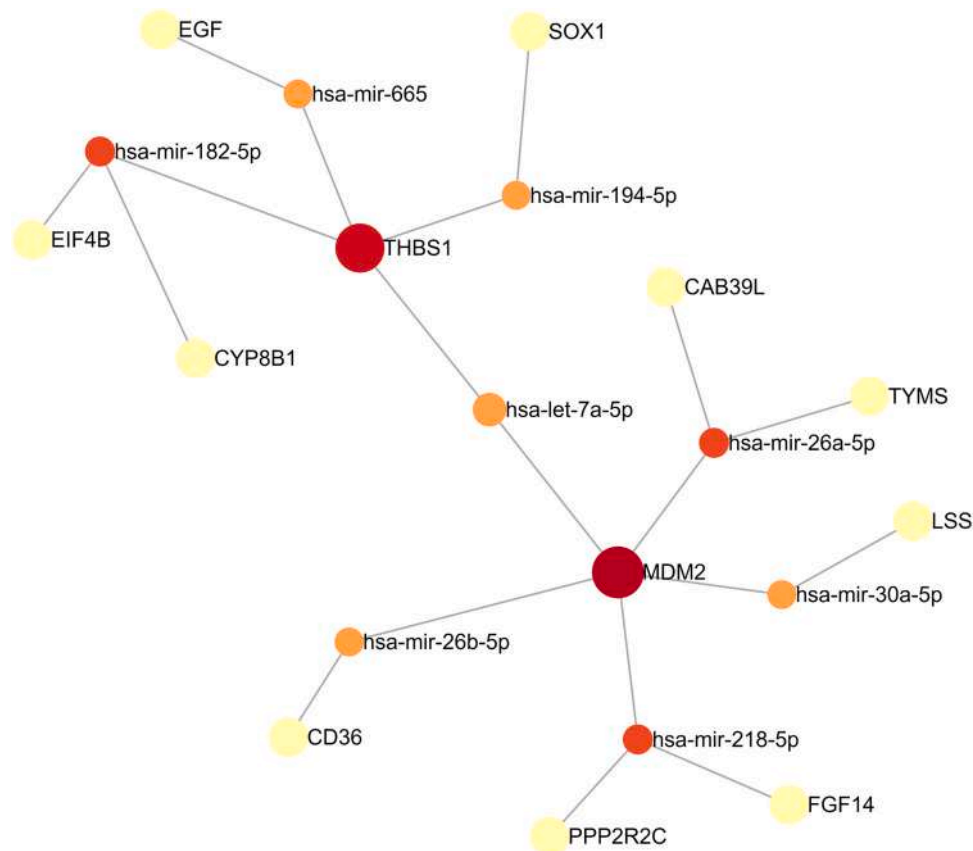


Fig. 7. miRNet miRNA-target interaction network of 12 differentially expressed genes and 8 miRNAs after pesticide exposure in cancer cell samples.

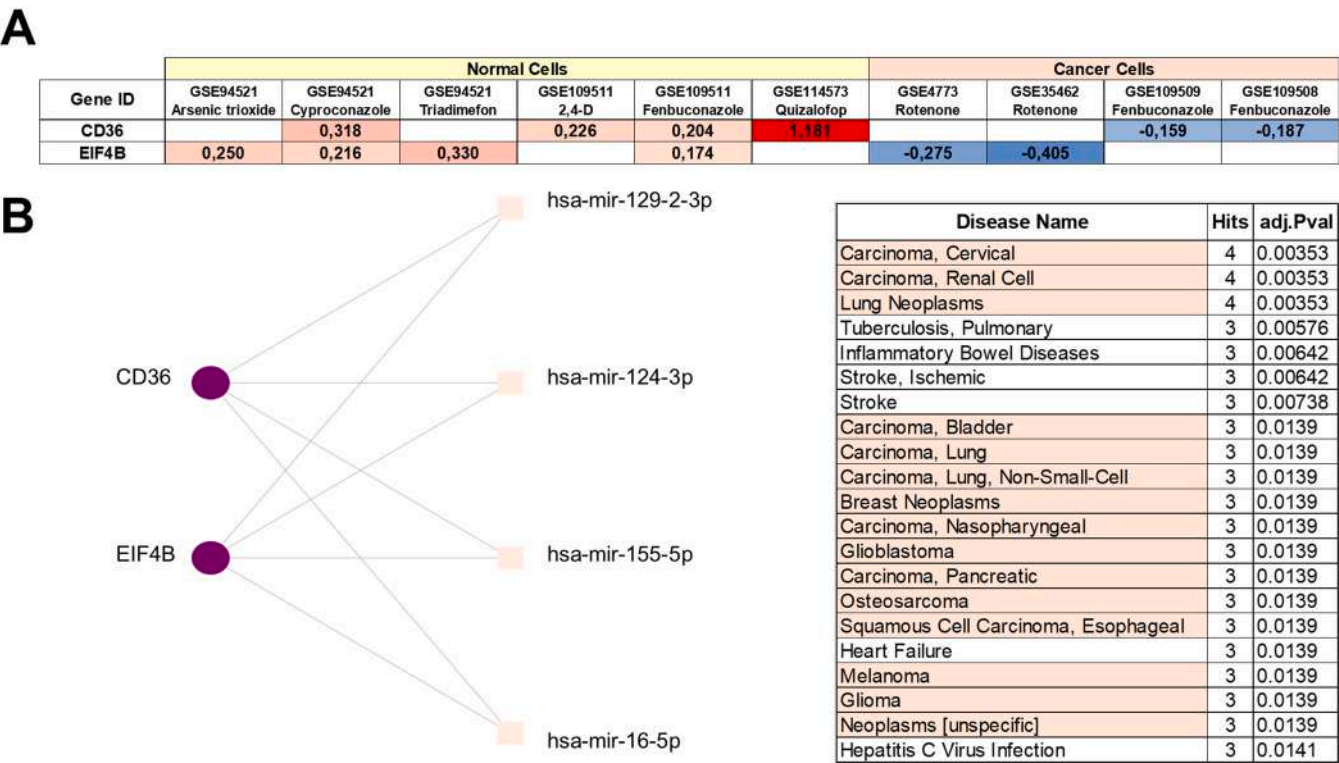
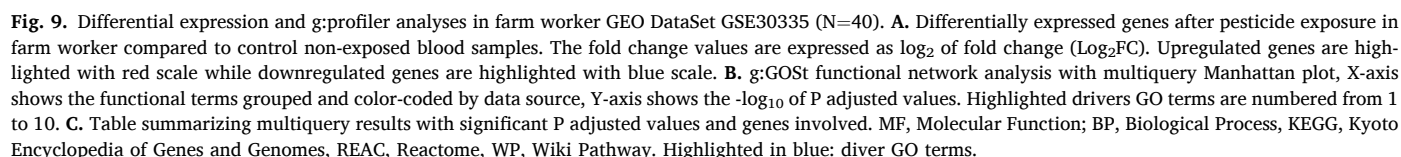


Fig. 8. Genes differentially expressed upon pesticide treatments in both normal and cancer cellular datasets. **A.** Following pesticide exposure, CD36 and EIF4B are significantly upregulated in normal cells (red scale) and significantly downregulated in tumour cells (blue scale). The fold change values are expressed as log2 of fold change. **B.** Left: MiRNet analysis highlights 4 miRNAs regulating both CD36 and EIF4B post-transcriptional levels. Right: MiRNet-miRNA-Disease table of diseases involving at least 3 out of 4 identified miRNAs. Highlighted in red in the table are cancer diseases.



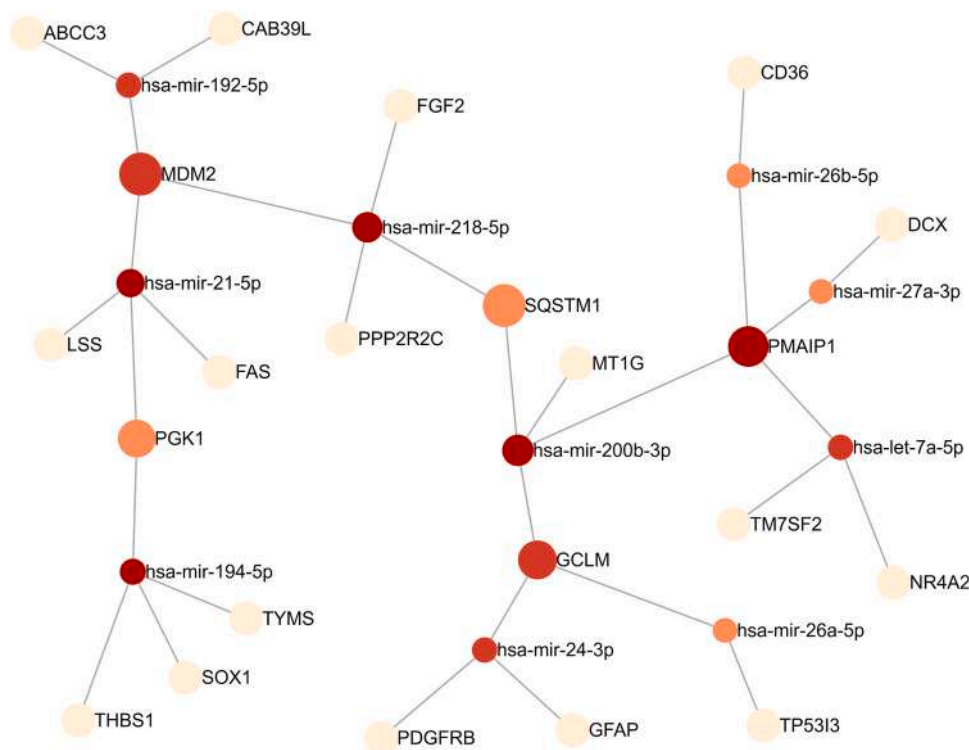


Fig. 10. miRNet miRNA-target interaction network of 22 differentially expressed genes and 10 miRNAs in farm workers after long-term exposure to pesticides.

ability to trigger the production of reactive oxygen species (ROS), causing oxidative stress and consequent damage to key biomolecules (including DNA, RNA, proteins and lipids) (Sule et al., 2022). The increase of oxidative stress is also linked with genotoxicity which could explain the pro-carcinogenic effects observed in cases of chronic exposure (Kapeleka et al., 2021).

Additionally, evidence from preclinical studies, including those associated with environmental contaminants such as pesticides, supports the role of environmental epigenetics in disease susceptibility. Among those mechanisms, which include DNA methylation and histone modifications, miRNAs have been largely studied. These have been found to be abnormal in samples from agricultural workers and other subjects in close and prolonged contact with pesticides (Valencia-Quintana et al., 2022).

Both genes and miRNAs represent suitable markers of pesticide exposure. They can be easily detected in blood cells; and miRNAs can be even detected as free circulating biomolecules, as well as in other biofluids (Giambò et al., 2021). In risk assessment, genetic and epigenetic biomarkers can be used to identify hazards, to assess exposure, as well as to associate a response with the probability to develop a given disease, such as cancer (Costa et al., 2020). Regarding genes, it is known that several are affected from pesticide exposure. For example, some DNA repair genes (XRCC1, OGG1 and XRCC4), metabolic enzymes (GSTP1 and PON1), and genes that respond to oxidative stress (SOD2 and NQO1) are all deregulated after pesticide exposure (Ceja Galvez et al., 2021). Unfortunately, genes are often non-selective biomarkers; therefore, the goal of this work was to uncover novel genetic biomarkers of exposure or effects that can be specifically correlated with the exposure to compounds belonging to the large family of pesticides.

High throughput transcriptomic technologies, including microarray profiling and whole-transcriptome RNA-seq, have been widely used in the latest 30 years to predict mechanisms of toxicity of molecules and determine their human relevance (Corton, 2019). We analysed all the latest 5-years published data regarding genes that are significantly affected by pesticide exposure and we identified a list of 70 genes that are involved in several biological processes, including homeostasis,

cellular response to chemical stimuli, regulation of metabolism, lipids adsorption and catabolism (Supplementary Fig. 1 and 2). A total of ten GEO DataSets of gene expression, including cellular samples and workers samples, were selected to perform differential analyses. Strikingly, depending on the nature of the samples, the set of significantly deregulated genes are only partially overlapping (Fig. 12B).

In normal cell samples, we found that GCLM, CD36, PMAIP1, EIF4B, SQSTM1, LPCAT4, CCND2, BMI1, TM7SF2 are significantly upregulated after pesticide treatment, while NR4A2, ABCB11, ABCC3, S100B are significantly downregulated. The pathways mainly affected by this set of genes are linked to xenobiotic transportation and metabolism, as well as to oxidative stress response. In particular, ATP binding cassette transporter genes ABCC3, ABCB11, have been widely investigated as putative biomarkers of pesticide exposure and effect, useful not only for human biomonitoring, but also for detection in fish, moths and other pests (Chedik et al., 2018; Hammer et al., 2020; Qi et al., 2016). Additionally, in line with our observations, other studies evidenced that both SQSTM1 and GCLM are upregulated in the presence of ROS and play critical roles in mitigating oxidative stress (Carroll et al., 2018; Yang et al., 2002). In particular, mutations in GCLM are already in study as putative indicators in pesticide risk assessment (Garshin et al., 2023).

In cancer cell samples, MDM2, LSS, EGF, TYMS were significantly upregulated, while SOX1, PPP2R2C, CD36, EIF4B, FGF14, THBS1, CAB39L, CYP8B1 were downregulated after pesticide exposure. The molecular pathways and biological processes involved in malignant cells are partially different from those observed in normal cells. In these samples, genes found significantly deregulated are mainly associated with lipoproteins, long-chain fatty acids and cholesterol binding or intracellular trafficking. Intriguingly, two genes, CD36 and EIF4B are differentially modulated following treatments in normal versus cancer cells. While in normal cells they are both upregulated, in cancer cells they are downregulated.

CD36 is a class B scavenger receptor involved in the modulation of cytotoxicity response associated with inflammation. The gene's product is a surface glycoprotein able to reduce ROS production after cellular damage (Cho et al., 2005). CD36 is a ubiquitously expressed receptor for

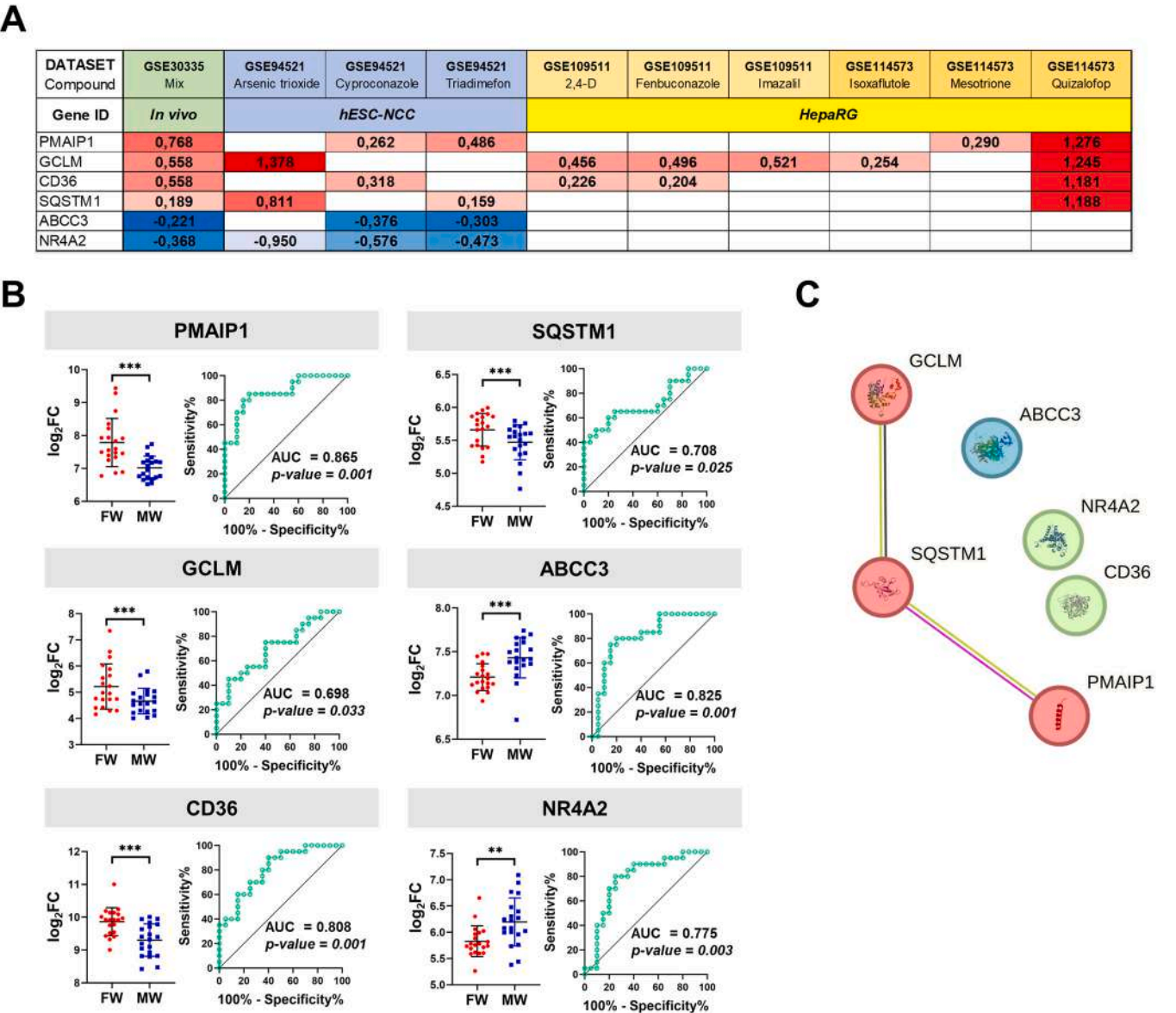


Fig. 11. Genes differentially expressed upon pesticide treatments in workers and normal cellular datasets. **A.** Following pesticide exposure, PMAIP1, GCLM, CD36 and SQSTM1 are significantly upregulated (red scale) and ABCC3 and NR4A2 are significantly downregulated (blue scale). **B.** log₂FC expression levels of single samples in GSE30335 DataSet and ROC curves with corresponding AUC for the 6 deregulated genes. **C.** STRING plot, with edges representing protein-protein associations (green line indicates association through text mining, black line indicates interaction due to co-expression, purple line indicates interaction experimentally determined). Node colour indicates differential clustering. ** p < 0.01; *** p < 0.001.

a plethora of ligands, such as low-density lipoprotein, long-chain fatty acids and the membrane of cells undergoing apoptosis. It has been demonstrated that this gene plays a dual role in normal and tumour cells; in normal cells, it attenuates angiogenesis by inducing apoptosis, whilst in tumour cells, it fuels metastasis and chemotherapy resistance by enhancing lipid uptake and fatty acid oxidation (Wang and Li, 2019). Also, when CD36 is expressed within non-transformed cells of the tumour microenvironment, such as cancer-associated fibroblasts or tumour-infiltrating T cells, it plays a main role through triggering immunosuppression and promoting cancer growth (Ao et al., 2023; Zhu et al., 2023). For all these reasons, in oncology it is considered an emerging prognostic marker and therapeutic target (Enciu et al., 2018; Ruan et al., 2022). EIF4B codes for a protein involved in translation initiation and it plays a main role in modulating responses of cellular survival and proliferation (Shahbazian et al., 2010). While its over-expression is correlated with oncogenic effect, the downregulation is associated with decreased viability and defective immune response

(Chen et al., 2021; Horvilleur et al., 2014). Pesticides impair cellular viability, therefore one possible explanation for the opposite CD36 and EIF4B levels observed in normal vs cancer cells, might be the fact that cancer cells are transformed and all the survival pathways are compromised (Hanahan, 2022). Importantly, as shown in Figs. 8A and 8B, looking at the four miRNAs (hsa-mir-129-2-3p, hsa-mir-124-3p, hsa-mir-155-5p, hsa-mir-16-5p) that regulate both gene expressions, they are found overexpressed in several cancers where they might downregulate CD36 and EIF4B following an insult, such as pesticide exposure (Dehghan et al., 2023). In particular, it has been observed that mir-129, mir-124, mir-155 have altered expression after exposure to organophosphates in animal models (Hong et al., 2019; Li et al., 2011; Miao et al., 2022). We hence performed the differential gene expression analysis in a dataset of blood cell samples from workers either exposed or not, to pesticides. The genes differentially regulated after chronic exposure to a mixture of pesticides are mainly involved in the regulation of cellular

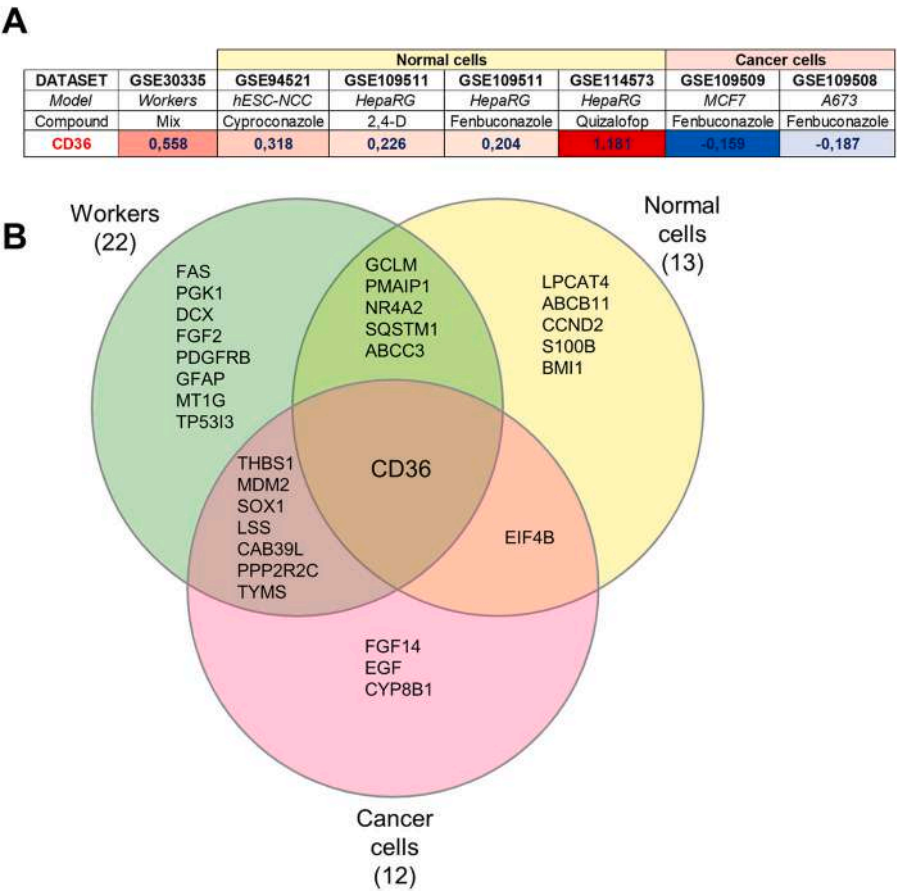


Fig. 12. Differential expression of CD36 gene after pesticide exposure in normal and transformed samples. **A.** CD36 is significantly upregulated in FW and normal cells datasets (red scale) after pesticide exposure, while it is significantly downregulated in tumour cells datasets (blue scale). **B.** Venn diagram showing deregulated gene lists in all ten GEO DataSets.

response to exogenous stimuli, including chemicals, and regulation of apoptotic response, phosphorylation and metabolic pathways. The outcome of the analysis partially overlapped with the other datasets with the other datasets. For what concerns non-transformed samples, 6 genes were commonly and significantly deregulated: PMAIP1, GCLM, CD36 and SQSTM1 upregulated; ABCC3 and NR4A2 downregulated. GCLM, SQSTM and PMAIP1 (a pro-apoptotic member of the Bcl-2 protein family) are functionally associated (Fig. 11C). Importantly, the results of ROC analysis, with high and significant AUC values suggested that all 6 differentially expressed genes can be considered as diagnostic discriminators between workers exposed or non-exposed to pesticides for long time (Fig. 11B).

Finally, CD36 has been found concurrently altered in all datasets analysed. The CD36 scavenger receptor is upregulated in normal samples (workers and cells) and downregulated in cancer cells. Intriguingly, CD36 was downregulated in cancer cells exposed to fungicide fenbuconazole. Marx-Stoelting et al. (2017), recently found that exposing mice with different genetic background may change the response in terms of CD36 expression levels, upregulated upon fungicide treatments only in wild-type animals (Marx-Stoelting et al., 2017). In contrast, other authors found that animals co-treated with organophosphates and a carcinogen show a gradual increase in expression of CD36 (Quintino-Ottomitar et al., 2023). Also, differences in post-transcriptional regulation of CD36 cells mediated by miRNAs in cancer compared to normal tissues might explain the diverse response to cellular toxicity induced by pesticides (Niculae et al., 2022).

5. Conclusions

In conclusion, our computational GEO DataSets differential expression analyses, conducted on *in vitro*, as well as in workers' samples, highlighted how exposure to different pesticides might lead to similar gene expression alterations. Six genes PMAIP1, GCLM, CD36, SQSTM1, ABCC3 and NR4A2 showed high and significant AUC predictive values in healthy individuals. These newly identified gene candidates can be further explored as useful biomarkers of exposure and effect. Among these genes, CD36 was upregulated in non-transformed samples and healthy workers, but downregulated in transformed cells after pesticide exposure, indicating a functional difference of response depending on the cellular background. Several miRNAs have been predicted as post-transcriptional regulators of these genes. Mechanistically, lipid metabolism and xenobiotic transportation were the mostly affected molecular pathways.

One real-world implication of this research would be to help to improve the effectiveness of pesticide toxicology assessment. Altogether, the present findings hold some promise to suggest novel gene candidates useful to stratify workers based on their individual susceptibility to detrimental effects of pesticides. Furthermore, preventive measures and assessment policies may be reinforced through the identification of workers at higher risk of developing pathologies linked to pesticide employment. An important next step will be to further validate the panel of genes in a large cohort of workers exposed to pesticides, as well as in workers with cancer possibly linked with occupational pesticide exposure.

Ethics approval and consent to participate

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CRediT authorship contribution statement

Concettina Fenga: Conceptualization, Data curation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Silvia Vivarelli:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Michele Teodoro:** Formal analysis, Validation, Writing – review & editing. **Sebastiano Italia:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Chiara Costa:** Validation, Visualization, Writing – original draft, Writing – review & editing. **Federica Giambò:** Formal analysis, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.etap.2024.104524](https://doi.org/10.1016/j.etap.2024.104524).

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