BIOMARKERS OF EXPOSURE, EFFECT AND SUSCEPTIBILITY TO ENVIRONMENTAL AND OCCUPATIONAL CHEMICALS

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BIOMARKERS OF EXPOSURE, EFFECT AND SUSCEPTIBILITY TO ENVIRONMENTAL AND OCCUPATIONAL CHEMICALS

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Biological Monitoring of Exposure to Benzene in Port Workers

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Port workers are exposed to a wide range of occupational hazards that can cause injuries and occupational diseases. Among these, exposure to benzene is one of the most important but least studied. The highest occupational exposures for port workers occur during the filling and loading of gasoline, and cleaning of tanks and receptacles. The aim of the study was to evaluate occupational exposure to low levels of benzene by measuring trans,trans-muconic acid (t,t-MA) in urine samples from workers operating at fuelling stations in a tourist port of Southern Italy. The overall sample was composed of 43 port workers of a tourist port in Southern Italy. In 2018, each participant provided two (morning and evening) urine samples for the determination of urinary t,t-MA. Urinary excretion of t,t-MA was always higher at the end of the work shift than at the beginning with significant difference ($p = 0.002$). In smokers, median t,t-MA urinary excretion is higher than non-smokers both at the beginning (90.5 μg/g creatinine vs. 61.45 μg/g creatinine) and at the end of the work shift (128.2 μg/g creatinine vs. 89.5 μg/g creatinine). Urinary excretion of t,t-MA is higher at the end of the work shift than at the beginning in both smokers and non-smokers, but the difference is significantly higher in non-smokers ($p = 0.003$) than in smokers ($p = 0.05$). In conclusion, our results showed that the role of inhaled benzene at fuelling stations in a tourist port can be relevant. On the basis of these results and the known adverse effects of benzene on human health, we encourage the use of personal protective equipment in the fuelling area of ports in order to minimize exposure to benzene to workers.

Keywords: port workers, benzene, biological monitoring, occupational exposure, t,t-muconic acid

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INTRODUCTION

According to the European Commission, seaports play an important role for economic development by promoting the European Union's external trade (90% of the total, in terms of weight) and internal market exchanges (40% of the total) (1). In the seaports of the 22 maritime Member States of the European Union, around 110,000 port workers are engaged in the loading and unloading of ships (2).

In the same way as other working sectors, port workers are exposed to a wide range of occupational hazards that can cause injuries and occupational diseases (3–7). They have a high risk of exposure to ergonomic hazards (e.g., repetition of movements, awkward positions), biological hazards (e.g., animals, microorganisms, bacteria, viruses, and fungi), physical agents (e.g., extreme temperatures, noise, vibrations, and radiation), psychosocial hazards (fatigue, irregular working hours, shift work, etc.), and chemical substances (8, 9). Furthermore, port workers may be exposed to asbestos in the course of their work (10). For a long time, asbestos was widely used in various fields (maritime, industrial, military and construction sectors, etc.), and the unaware use of this mineral caused adverse effect on human health and the environment (11–14).

Many industrial, agricultural, and medical organizations use hazardous substances (15–17). In this field, the main operations that expose port workers to contact/inhalation of harmful chemicals are as follows: mechanical maintenance, cleaning and sanitizing of ship interiors, unloading of raw materials from the holds of ships and/or loading of finished products, storage of chemicals, storage and transport of vehicles, and refueling of ships at gasoline stations. Actually, port workers can be exposed to different types of toxic agents such as carbon monoxide, volatile organic compounds (e.g., benzene), nitrogen oxides, sulfur oxides, particulate matter, metallic elements, and pesticides. Other chemicals produced by port activities are formaldehyde, polycyclic aromatic hydrocarbons, and dioxins (18–21). Many of these have mutagenic and/or carcinogenic effects such as benzene, one of the most toxic environmental and occupational pollutants (22). Exposure to benzene usually occurs in a wide variety of occupational fields. In particular, this toxin is produced from chemical plants, oil refineries, petrochemical industries, coke production plants, hazardous waste landfills, and petrol service stations (23, 24). Benzene is also present in living environment, released by cigarette smoking and vehicles exhausting fumes (25). According to the European Chemical Agency (ECHA), the highest occupational exposures occur during the filling and loading of gasoline, and cleaning of tanks and receptacles (26). Because of its known carcinogenic properties (acute myeloid leukemia—acute non-lymphocytic leukemia), the International Agency for Research on Cancer (IARC) has classified benzene as carcinogenic for humans (Group 1) (27).

Benzene is highly volatile, and occupational exposure occurs mainly by inhalation, although dermal exposure is possible in some specific conditions such as immersion of the skin in solution or when the airborne concentration of benzene is very low (28, 29). Following exposure, benzene is partially eliminated in the exhaled air (17%); the remaining part is metabolized and excreted in the urine in the form of phenolic compounds (e.g., phenol, hydroquinone, catechol, and trihydroxybenzene), S-phenylmercapturic acid (S-PMA), trans,trans-muconic acid (t,t-MA), and unmetabolized benzene (U-benzene) (30, 31). Biological monitoring of benzene exposure involves the measurement of benzene levels or its metabolites in the biological samples. Suggested biomarkers for benzene exposure in occupational settings are urinary samples of unmetabolized benzene or S-PMA (32, 33). For the purpose of biomonitoring to low concentrations of benzene, the used biomarker is the t,t-MA. This biomarker is a urinary metabolite of benzene that is used in routine practice because it is a reliable and relatively convenient biomarker (34–36).

Italian Legislative Decrees 152/2006 and 66/2005 regulate, for the purposes of the prevention and limitation of atmospheric pollution, the characteristics of marine diesel and establish that the benzene content must be <1.0% (v/v) and that of total aromatic polycyl hydrocarbons must be lower than 35% (v/v). Italian Legislative Decree 155/2010 established the Occupational Exposure Limit (OEL) for benzene of 1.6 mg/m^3, with a Short-Term Exposure Limit (STEL), equivalent to 15-min average exposure, of 8 mg/m^3. The EU is preparing for a much lower OEL. In 2017, the ECHA suggested a new OEL of 0.1 ppm, or 0.3 mg m^{-3}, and a year later (2018), the ECHA RAC proposed an even lower OEL of 0.05 ppm, or 0.16 mg m^{-3} (33).

For the purpose of biomonitoring to low concentrations of benzene, one of the most used biomarkers is the t,t-MA. This well-known and relatively convenient biomarker is a urinary metabolite of benzene (34–36). However, t,t-MA levels in urine are influenced by other factors such as cigarette smoking and sorbic acid (food preservative) introduced with diet (24).

The American Conference of Governmental Industrial Hygienists (ACGIH) established a Biological Exposure Index (BEI) of 500 μg t,t-MA/g creatinine in urine for occupational benzene exposure (equal to 0.75 μg/ml with respect to an excretion of 1.5 g/L creatinine in urine) (37).

Although several biomonitoring studies have been conducted to assess occupational exposure to benzene in different group of workers (23, 24, 38), there are no reports in literature on biological monitoring in port workers at gasoline stations. The aim of this study was to evaluate occupational exposure to low levels of benzene by measuring t,t-MA in urine samples from 43 workers operating at fuelling stations in a tourist port of Southern Italy.

MATERIALS AND METHODS

Study Population

The overall sample consisted of 43 male port workers, and the selection was carried out on a random basis. The work activities consisted of refueling pleasure boats. Data regarding personal characteristics, length of service, and smoking habit were collected by technical personnel. All subjects had given written informed consent to take part in the study, after having received a full explanation of the aims and the methods.

Biological Monitoring

The overall sample was composed of 43 port workers potentially exposed to low levels of benzene. Urine sampling was performed between April 2018 and June 2018.

Each participant provided two (morning and evening) urine samples for determination of urinary t,t-MA. The first sampling was conducted in the early morning (the first urination of the day), and the second sample was collected at the end of the

8 h work shift with a 6-day week. Urine samples were collected in 10-ml polystyrene single-use containers and were frozen at −20°C until analysis. The benzene metabolite t,t-MA is widely used as a biological indicator of exposure to this xenobiotic. The analysis of t,t-MA was performed by high-performance liquid chromatography (HPLC) with UV detection method, using a commercial laboratory kit (Chromsystems Instruments &Chemicals GmbH, Grafelfing, Germany) (39). Briefly, 750 μl of the internal standard was put into a reaction vial and mixed using a vortex. The sample (250 μl of urine) preparation is based on the efficient and selective purification with solid phase extraction. This includes the addition of an internal standard (provided by the manufacturer) to the sample with a simultaneous pH adjustment and subsequent transfer to the SPE column. Sequenced washing steps (with buffer 2 and 3 provided by the manufacturer) are then performed to eliminate interfering substances. The limit of quantification is 0.02 mg/L, the linearity is 0.02 up to 10 mg/L, the recovery is between 93% and 98%, the intra-assay coefficient of variation (CV) is <1.5–1.7%, and the interassay CV is <2.9–3.4%. Internal quality was secured by the Shewhart control cards and by the application of the Westgard Rules. The external control is assumed by the matrix analysis with known concentration.

Finally, the t,t-MA is eluted and stabilized simultaneously. This analysis method is very sensitive and allows us to determine concentrations over 20 μg/L. Urinary creatinine was also quantified to check the acceptability of each urine sample and to make appropriate corrections to some of the biomarkers measured. Urinary creatinine range, according to WHO criteria, should be between 0.3 and 3 g/L (40). Urinary creatinine concentrations were measured using a fully automated clinical chemistry analyzer (Cobas® 6000 Modular Analyzer, Roche Diagnostics, Basel, Switzerland, Europe). Internal control is ensured by daily checks provided by the manufacturer, while external control is ensured by the participation of interlaboratory circuits organized by the Sicilian Region.

A diet (free of cheese, yogurt, industrial sweets, dried fruit, fruit preparations, canned food, fizzy drinks) was prescribed to workers during the entire sampling period in order not to influence the study results. T,t-MA is also a metabolite of sorbic acid, which is a substance naturally contained in some foods or used as a food preservative.

Statistical Analysis

A preliminary statistical analysis of the data was performed using STATA 12 software (41). Comparisons were performed with Student's *t*-test for independent samples (smokers vs. non-smokers) and for paired samples too (beginning of the shift work vs. end of the shift work). The criterion for significance was set at $p < 0.05$. For descriptive analysis (media, median, and range), results are presented as μg/g creatinine.

RESULTS

Study Population

The adherence rate to the study was 100%. **Table 1** shows the general characteristics of the study population. The overall sample consisted of 43 male port workers ranged in age from 21 to 64 years and with a mean working life of 19.63 years. Workers were divided into two groups: 15 smokers (average age 48 years old and mean working life of 21.4 years) and 28 non-smokers (average age of 47.07 years old and mean working life of 18.68 years). The comparison between the group of smokers and non-smokers did not show significant differences for age, BMI, and years of service. With regards to smokers, the average number of cigarettes smoked per day was 16.33, and the mean number of years as a smoker was 29.73.

TABLE 1 | General characteristics of study population, n°43.

	Overall (N°43)	Smokers (N°15)	Non-smokers (N°28)
Age (Years)			
Mean ± SD	45.44 ± 9.93	48 ± 10.13	47.07 ± 9.73
Median	47	49	44
Range	21–64	21–64	24–62
BMI (Kg/m^2)			
Mean ± SD	21.61 ± 1.87	21.69 ± 1.81	21.57 ± 1.93
Median	21.8	21.8	21.7
Range	17.6–25.1	18.6–25.1	17.6–25.1
N° Cigarettes/day	N/A		N/A
Mean ± SD		16.33 ± 6.4	
Median		15	
Range		10–30	
N° of Years as Smoker	N/A		N/A
Mean ± SD		29.73 ± 8.88	
Median		29	
Range		6–46	
Length of service—years			
Mean ± SD	19.63 ± 9.55	21.4 ± 10.45	18.68 ± 9.09
Median	20	22	19
Range	2–41	2–41	2–41

Biological Monitoring

The urinary t,t-MA was detected as a marker of the internal dose of benzene exposure. **Table 2** shows the results of the analysis of t,t-MA urinary excretion at the beginning and at the end of the work shift in the overall sample of workers, divided for smoking and non-smoking habit. The results were subdivided by smoking habit because reports in literature have demonstrated that cigarette smoke influences urinary excretion of this metabolite (42). In the overall sample, the median value of t,t-MA urinary excretion was 79.8 and 102.7 μg/g creatinine at the beginning and the end of the work shift, respectively. At the beginning of the work shift, the lowest concentration of t,t-MA was 17.5 μg/g creatinine in non-smokers, and the highest was 231 μg/g creatinine in smokers. At the end of the work shift, the

TABLE 2 | T,t-muconic acid urinary excretion.

	Overall (N°43)	Smokers (N°15)	Non-smokers (N°28)
T,t-muconic acid urinary excretion			
Beginning of shift work			
Mean			
μg/g creat	81.94 ± 46.28	116.59 ± 50.03	63.39 ± 31.78
Median			
μg/g creat	79.8	90.5	61.45
Range			
μg/g creat	17.5–231	52.3–231	17.5–124.9
T,t-muconic acid urinary excretion			
End of shift work			
Mean			
μg/g creat	117.52 ± 58.91*	153.19 ± 65.64	98.41 ± 45.59**
Median			
μg/g creat	102.7	128.2	89.5
Range			
μg/g creat	23.9–325.4	87.4–325.4	23.9–189.6

*$p = 0.002$; **$p = 0.003$: comparison between mean t,t-MA urinary excretion at the beginning of the shift work and at the end of the shift work.

lowest concentration of t,t-MA was 23.9 μg/g creatinine in non-smokers, and the highest was 325.4 μg/g creatinine in smokers.

Urinary excretion of t,t-MA was always higher at the end of the work shift than at the beginning with significant difference ($p = 0.002$) (**Figure 1**); this difference is stronger in non-smokers ($p = 0.003$) than in smokers ($p = 0.05$) (**Figure 2**). In smokers, median t,t-MA urinary excretion is higher than non-smokers both at the beginning (90.5 μg/g creatinine vs. 61.45 μg/g creatinine) and at the end of the work shift (128.2 μg/g creatinine vs. 89.5 μg/g creatinine).

DISCUSSION

According to the International Labour Organization (ILO), the main health hazards related to port operations are, among others, fumes, dust, and exposure to hazardous chemical substances (29). These substances can cause serious health effects in exposed individuals (43). Despite the multitude of harmful substances that may be present in port areas, there are limited pieces of information about biological monitoring and implications of chemical risk in port workers.

The results of our study showed that the levels of t,t-MA in urine samples taken from all workers (smokers/non-smokers) at the end of the shift had significantly higher values compared with those measured at pre-shift. The median value of t,t-MA urinary excretion in smoking workers is higher both at the beginning and at the end of the shift than in non-smoking workers. The urinary concentration of t,t-MA never exceeded the BEI of ACGIH 500 μg t,t-MA/g creatinine, in both smokers and non-smokers.

In agreement with our results, a recent study by Forsell et al. (44) explores benzene exposure in deck crews on tankers investigating the correlations between benzene exposure and benzene in alveolar air, benzene in urine, and t,t-MA in urine. They found that the average 4-h benzene exposure level for those exposed was 0.45 mg/m^3 and for those non-exposed 0.02 mg/m^3. All the biomarkers were significantly higher in post-shift samples among exposed and correlated with the exposure level.

Furthermore, Davenport et al. (45) conducted a study in an attempt to quantify short-term exposure levels to Coast Guard personnel performing routine inspection activities aboard commercial tank barges carrying gasoline. A total of 43 personal and 68 area samples were analyzed for benzene. Although none of the personal samples met or exceeded proposed or established short-term exposure standards, many of the area sampling results indicated that a significant risk of acute exposure exists in the vicinity of valves, pressure lines, and connections. Fakhrinnur et al. (38) measured t,t-MA levels in a group of 33 service station workers and found a significant correlation between the duration of filling the fuel and the level of t,t-MA in urine ($p = 0.000$). Also, unlike our results, seven workers had urine tt-MA levels that exceeded the value of BEI set by ACGIH (500 μg/g creatinine).

In our study, biomonitoring of benzene exposure was assessed by measuring one of its urinary metabolites, the t,t-MA, a reliable biomarker for low-level benzene exposure. T,t-MA is an indicator used for routine biological monitoring, mainly due to the analytical method for determining its concentration (HPLC-UV), practicable in all industrial and environmental toxicology laboratories (46, 47).

Among factors affecting urinary t,t-MA detection is the sorbic acid (additive in food, cosmetics, and pharmaceuticals), and therefore, workers were subjected to an elimination diet during the sampling period. In humans, after ingestion of a single dose of 447 mg sorbic acid and, during 2-day trials, ingestion of three doses of 1 mg sorbic acid/kg body weight, it was found that about 0.05–0.51 and 0.15–0.34%, respectively, of the dose was converted into t,t-MA. Weaver et al. found that in subjects who consumed two sorbic acid–preserved foods, a great increase in t,t-MA urinary concentrations was observed with individual peaks ranging as high as 705 μg/g creatinine (48, 49).

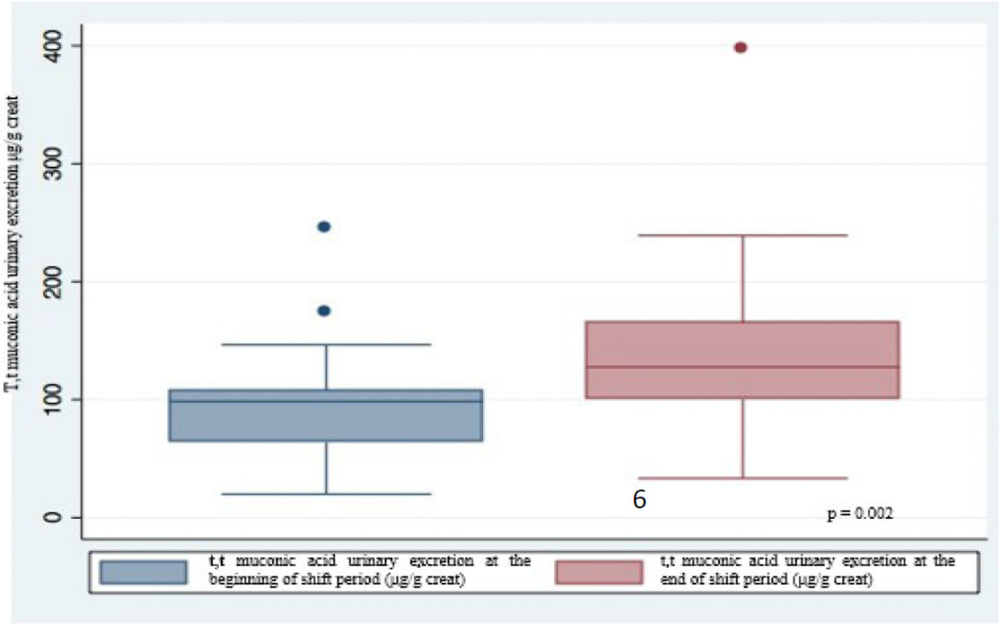

FIGURE 1 | Urinary excretion of t,t-muconic acid at the beginning and at the end of the shift work.

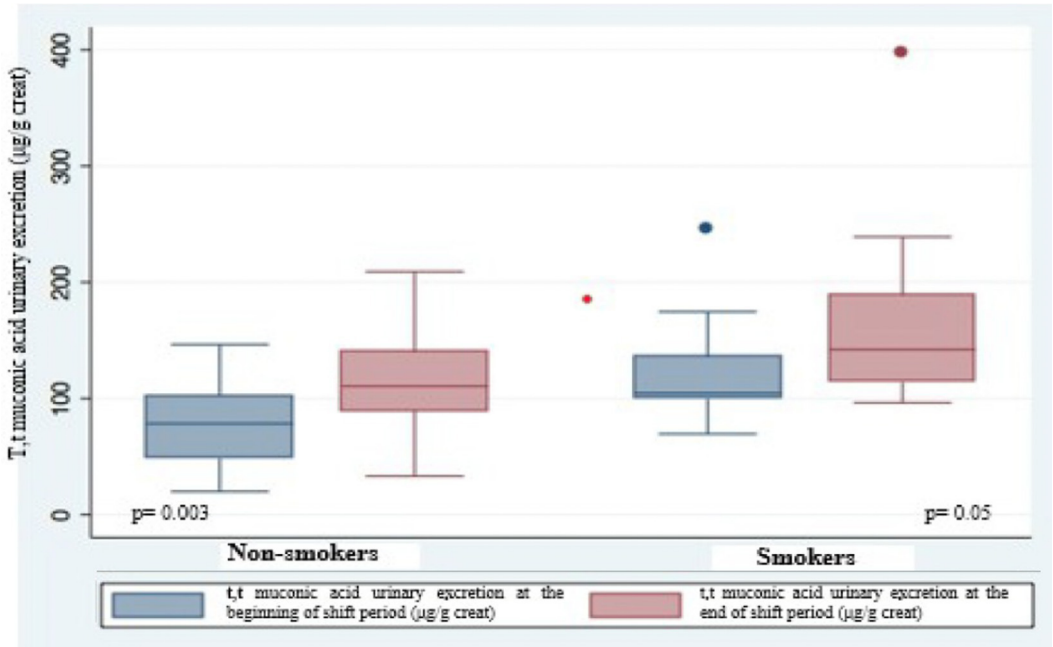

FIGURE 2 | T,t-muconic acid urinary excretion in smokers and non-smokers.

Our study has some limitations. We have no information about the use of personal protective equipment (PPE) and a not very large sample. In addition, we do not have data relating to the actual duration of the refueling activity during each single working shift and during the entire working week, because this activity is variable depending on the demand. On the other hand, our study also has strengths. The main one is the systematic biological monitoring, carried out through the determination of t,t-MA in morning (first urination of the day) and evening urine samples, which allowed us to obtain valuable information about the exposure to benzene of each port worker.

In conclusion, the increase in urinary excretion of t,t-MA from the beginning to the end of work shift in exposed non-smoking workers showed that the role of

inhaled benzene at fuelling stations in a tourist port can be relevant. These findings are important considering the forthcoming OEL reduction. If, in fact, in the past, port workers were considered exposed to low doses of benzene, almost always below the OELs, in the near future, this will no longer be true. As a consequence, it will become increasingly important to monitor benzene exposure in order to keep it below new OELs and avoid adverse effects on workers' health.

On the basis of these results, we encourage the use of PPE in the fuelling area of ports in order to minimize exposure to benzene. In addition, port workers should undergo pre-placement and periodic medical examinations in order to identify health problems caused by exposure to benzene.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CL, EC, VR, and LV: conceptualization. CL, EC, and VR: investigation and data curation. GF and DC: formal analysis and methodology. LV: supervision. LD, AC, FM, EC, SS, and FB: writing—original draft. LV, LD, AC, FM, EC, SS, and FB: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Accidental Use of Milk With an Increased Concentration of Aflatoxins Causes Significant DNA Damage in Hospital Workers Exposed to Ionizing Radiation

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The occupational exposure to ionizing radiation (Irad) or associated with mycotoxin-contaminated food may lead to genome damage and contribute to health risk. DNA damage in 80 blood samples of hospital workers occupationally exposed to low—doses of Irad was compared with 80 healthy controls. Among them, 40 participants accidentally consumed milk with increased concentration of Aflatoxin. All participants underwent the testing for micronuclei from blood, and 40 of them 8-OHdG from urine. The frequency of micronuclei (MN) was analyzed by cytokinesis-block peripheral blood lymphocytes and the level of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) by ELISA. The Irad led to increased frequency of MN ($p < 0.05$) and 8-OHdG level at exposed hospital workers. The consumption of milk with increased concentration of aflatoxin probably raised MN frequency and 8-OHdG value. Higher consumption of aflatoxin-contaminated milk (≥2 L/monthly) caused significantly increased MN frequency and 8-OHdG value in comparison to lower milk intake (≤0.5 L/monthly). Also, confounding factors, such as age, gender, and smoking status of all participants were included in the study. The obtained results revealed an increased incidence of MN and 8-OHdG level among hospital workers exposed to low-doses of IRad and milk with increased aflatoxin concentration.

Keywords: aflatoxin, genotoxicology, ionizing radiation, occupational exposure, 8-OHdG, hospital workers, ELISA, micronuclei

HIGHLIGHTS

- Determination of DNA damage among hospital personnel after accidental consumption of milk in the period characterized by increased concentration of aflatoxin.
- Increased frequency of micronuclei and the level of 8-OHdG were noticed in participants exposed to Irad and aflatoxin-contaminated milk.
- Consumption of milk with increased aflatoxin content contributes to DNA damage.

INTRODUCTION

Ionizing radiation (IRad) is a major stress factor that may induce cell damage and, consequently, carcinogenesis (1). IRad acts directly or indirectly via radiolysis of water, thereby creating a reactive oxidative species (ROS). ROS can attack nucleic acids, which is followed by many different types of DNA damages. DNA damage may occur as a result of the impact of IRad oxygen radicals generated during the endogenous process, as well as those from working and living environment. Therefore, it is very important to assess the absorbed dose of IRad in persons who are occupationally exposed to IRad in compliance with relevant legislation.

In combination with the occupational exposure to IRad, eating habits during particular period of life may also contribute to additional genome damage. Consuming food containing high levels of mycotoxins, which are associated with certain disorders in both humans and animals, causes significant health risk.

Aflatoxins (AFT), well-known mycotoxins and highly toxic metabolites are produced by *Aspergillus* species, mainly *A. flavus* and *A. parasiticus* (2). According to the International Association for Research on Cancer (IARC) classification, AFB_1, AFB_2, AFG_1, and AFG_2 are group 1 carcinogens, whereas AFM_1 is a group 2B carcinogen (3).

The AFB1, known DNA-damage agent, bonds covalently its metabolites (AFB1-−8,9-epoxide) to DNA in target cells, which results in AFB1-N7-guanine adducts, consequently leading to mutations and tumorous changes at different organs (4).

In terms of cytotoxic effect, AFB1 leads to lipid peroxidation and oxidative stress in hepatocytes and inhibits nucleotide phosphodiesterase cyclical activity in tissues (5). Aflatoxin-B1 and -M1, its main monohydroxilated metabolite, enter into the body of domestic animals through contaminated plant food and milk products consequently become an indirect source of AFTs.

The majority of developed countries established maximum legally permitted residue levels (MRL) for AF M1 in milk. In the European Union, MRL for AF M1 is 0.05 μg/kg milk or milk-based product (6). In Serbia, MRL for AFM1 (7) is harmonized with the values set out by European Union (EU) Regulation.

The published studies from Mediterranean and Middle East countries indicate that environmental conditions, especially warm and dry weather, may favor the occurrence of AFTs in agricultural products and therefore AF M1 in milk (8). Low contamination frequency of AF M1 in the reports from Serbia for the period before AFT contamination can be explained by the lower rate of AFTs in maize and other feed material in Serbia due to optimal weather conditions in these years (8).

The extreme warm and dry weather conditions in 2012 led to contamination of agricultural crops and elevation of AFT B1 in animal feed used for feeding lactating animals in Serbia and the region- almost 80% of the total production of maize in 2012 was estimated to be affected by aflatoxins, which caused large-scale crisis in February 2013, when AFM1 was detected in milk produced by Serbian dairy companies (9, 10).

Two additional studies indicated serious risk to consumers since AF M1 levels exceeding EU MRL (at concentration range from 0.05 to 0.98% μg/kg) were detected in 80–100% of different milk samples collected between February and June 2013 on the Serbian market (11, 12).

Therefore, taking into account geno- and cytotoxic properties of AF M1, milk consumption as a monitored parameter was also included in our investigation.

During the last decade, the Cytokinesis-Blocked Micronucleus Assay (CBMN) has become a thoroughly validated and standardized technique for the evaluation of DNA damage at individuals occupational, medical and accidentally exposed to radiation. It is known that increased MN frequency in peripheral blood lymphocytes represents a predictive biomarker of cancer risk (13). As a part of nutrigenetics, CBMN assay is used to determine the influence of dietary habits on the changes in the human genome (14).

Also, 8-OHdG, the repair product of excision enzymes which is excreted through urine is used as a biomarker to assess the extent of oxidative DNA damage and repair in the occupational setting (15).

To date, there is no information on conducting risk assessment for two agents with different mechanisms of action (i.e., energy deposition from ionizing radiation vs. DNA interactions with chemicals). In our investigation, we examined the influence of low-dose IRad and consumption of milk in the period when over 80% samples from Serbian market had aflatoxin concentrations >0.05 μg kg^{-1} on the aforementioned biomarkers of DNA damage. Our study was conceived to monitoring similar biological endpoints for determining genetic hazard, micronuclei in peripheral blood and 8-OHdG in urine. The analysis was conducted on medical workers chronically exposed to IRad and the results were compared with the unexposed control groups. The subjects were also divided into the sub-groups according to age, gender and smoking status. The combination of monitored biomarkers could give more complete view of the influence of both IRad and consumption of AF M1-contaminated milk on occupationally exposed persons and thus provide information about their cumulative health risk associated with carcinogenesis.

EXPERIMENTAL PROCEDURES

Group Description

This retrospective study included 160 participants-−80 healthy volunteers and 80 hospital workers chronically exposed to low doses of ionizing radiation, employed at the Oncology Institute of Vojvodina and the Institute for Lung Diseases of Vojvodina, Republic of Serbia. Various equipment was the source of occupational radiation exposure among bronchoscopy and radiotherapy medical personnel. The workers in bronchoscopy unit performed, wearing lead aprons, up to 10 interventions per day, and X-ray source was active to 2 min during bronchoscopy procedure, and the ones from radiotherapy unit were in a

Abbreviations: OHdG, 8-hydroxy-2′-deoxyguanosine; AFT, aflatoxin; CBMN, Cytokinesis-Blocked Micronucleus Assay; DNA, Deoxyribonucleic acid; ELISA, The enzyme-linked immunosorbent assay; FCS, fetal calf serum; IARC, International agency for research on cancer; IRad, ionizing radiation; MN, micronuclei; MRL, maximum residue level; RNS, Reactive nitrogen species; ROS, reactive oxygen species.

control room, protected from direct source of ionizing radiation. Persons who had medical treatment, radiography, or vaccination within the previous 9 months were not included in the study. The questionnaire filled by each participant included general information about professional exposure to Irad and about life habits like smoking, alcohol consumption, medical history, drug intake and diagnostic medical irradiation. Blood samples from all participants were collected during 2012, until June 2013. In addition to blood samples for micronuclei test during the first half of 2013, urine samples were collected for 8-OHdG, and participants filled in the questionnaire on habits regarding milk consumption. The study was approved by the Ethics Committee of the Institute for Lung Diseases of Vojvodina and informed consent was obtained from participants.

Cytokinesis Block Micronucleus Test (CBMN)

Heparinized whole blood was collected by venous puncture from participants and used for the peripheral blood lymphocyte cultures in CBMN test. Briefly, 0.5 ml of the whole blood was added to 5 ml of RPMI 1640 cell culture medium (Sigma, USA) supplemented with 2 mM glutamine, 20% of heat-inactivated fetal calf serum (FCS, NIVNS, Serbia) and antibiotics: 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN, Serbia). Cell cultures were stimulated for division with phytohemagglutinin (PHA-M, Sigma, USA) at a final concentration of 20 μg/mL and incubated at 37°C for 72 h in 5% CO_2 atmosphere with 95% humidity.

CBMN was performed applying standard cytogenetic procedure with minor modifications regarding staining (1). Forty-four hours after stimulation of the lymphocyte culture with PHA, cytochalasin-B (Sigma, USA) was added at final concentration of 6 μg/mL. After 72 h, the cells were collected by centrifugation, exposed to a cold 0.075 M KCl hypotonic solution and fixed three times. The first fixation was in methanol-acetic acid (3:1) with 1% formaldehyde, where the two following fixations were in methanol—acetic acid (3:1) alone. Drops of a concentrated cells suspension were placed on dried slides. Cells were stained with Giemsa (2%) in distilled water with three drops of NH_4OH for 9 min.

At least 1,000 cells per each sample were analyzed. Monitored values included: frequency of micronuclei, micronucleus distribution and proliferation index. MN frequency was presented as a number of micronuclei per 1,000 examined binuclear cells. Micronucleus distribution was acquired by scoring the binuclear cells containing one or more micronuclei. The proliferation index (PI), which represents a measure of the number of cell cycles that a cell population passes through, was calculated according to the formula:

$$\mathrm{NDI} = \mathrm{M1} + 2\mathrm{M2} + 3(\mathrm{M3} + \mathrm{M4})/\mathrm{N}$$

where M1–M4 represents the numbers of cells with 1–4 nuclei, respectively, and N is the total number of scored cells (1). The prepared material was observed and analyzed by light microscopy (Olympus BX51, Germany).

8-Hydroxy-2′-Deoxyguanosine (8-OHdG)

Determination of 8-OHdG level was conducted according to the commercial enzyme-linked immunosorbent assay kit (highly—sensitive 8-OHdG check; Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd., Shizuoka, Japan). The urinary concentration of 8-OHdG was expressed by creatinine to avoid the effect of urine volume fluctuation [8-OHdG (μg/ml): creatinine (g/ml)] = 8-OHdG (μg/g creatinine). For the determination of urinary creatinine concentrations a modified Jaffe's method was used (16).

Statistics

To obtain the differences between the observed groups relative to micronuclei frequency and 8-OHdG values, the results were analyzed by Wilcoxon Matched Pairs Test, Mann Whitney U test and ANOVA, using STATISTICA Release 12. After adjusting for potential confounding factors (age, gender, and smoking status), multivariate analysis was performed by ANCOVA to assess the differences in micronuclei and 8-OHdG among the study groups. Adjustment for multiple testing was carried out by a *post-hoc* LSD test. The statistical significance for all tests was set at $p < 0.05$.

RESULTS

The Characteristics of the Subjects Included in the Study

The characteristics of both control and the group exposed to Irad are shown in **Table 1**. Regarding to the period of sample collection and milk consumption habits, both groups are divided to sub-groups: "with AFT"-participants who consumed the milk with increased concentration of AFT and "without AFT"-participants who didn't consume the milk with increased concentration of AFT.

Effect of Ionizing Irradiation on Micronuclei and 8-OHdG in Hospital Workers

The analysis of micronuclei (**Figure 1A**) and 8-OHdG (**Figure 1B**) revealed that hospital workers exposed to ionizing irradiation had higher values than unexposed healthy voluntairs from the control group. The difference between MN frequency in a group exposed to Irad and control group were significant (MN: $p < 0.05$; Wilcoxon test) (**Figure 1A**).

The Effects of Milk Consumption on Micronuclei and 8-OHdG

In both control group and the group exposed to Irad, participants who consumed a milk with AFT had higher frequency of micronuclei than those who didn't use contaminated milk.

Statistical analysis revealed that MN frequency in workers exposed to Irad who consumed milk with AFT was significantly higher in comparison to workers who didn't ($p = 0.015$; *post-hoc* LSD test), as well as in comparison to unexposed volunteers who also didn't use contaminated milk ($p = 0.006$; *post-hoc* LSD test) (**Figure 2**).

The groups "with AFT" were divided into two sub-groups according to the amount of consumed milk during the previous

TABLE 1 | Characteristics of subjects included in the study.

Variables		Control ($n = 80$)		Irad ($n = 80$)	
		Without AFT ($n = 70$)	With AFT ($n = 10$)	Without AFT ($n = 50$)	With AFT ($n = 30$)
Age (years)	Median	36	44	42	46.5
	Range	22–68	26–65	24–61	28–62
Young	22–45 years	47	5	30	14
Old	46–62 years	23	5	21	16
Gender	Male	27	2	21	4
	Female	43	8	29	26
Exposure time to ionizing radiation	Median	nn	nn	na	16.50
(years)	Range	nn	nn		2–36
Smoking status (%)	Non-smokers	52.85	80	21	63
	Smokers	47.14	20	29	37
Duration of smoking (years)	Median	na	16	na	26
	Range		7–25		10–39
Nutrition (%)	Vegetarian	0	0	0	0
	Non-vegetarian	100	100	100	100
Milk consumption	≥2 L/month	na	6	nn	19
(Number of individuals)	≤0.5 L/month		4		11
Irradiation during last month (mSv)	Median	nn	nn	na	0.21
	Range	nn	nn		0.16–2.05

Na, not available; nn, not necessary.

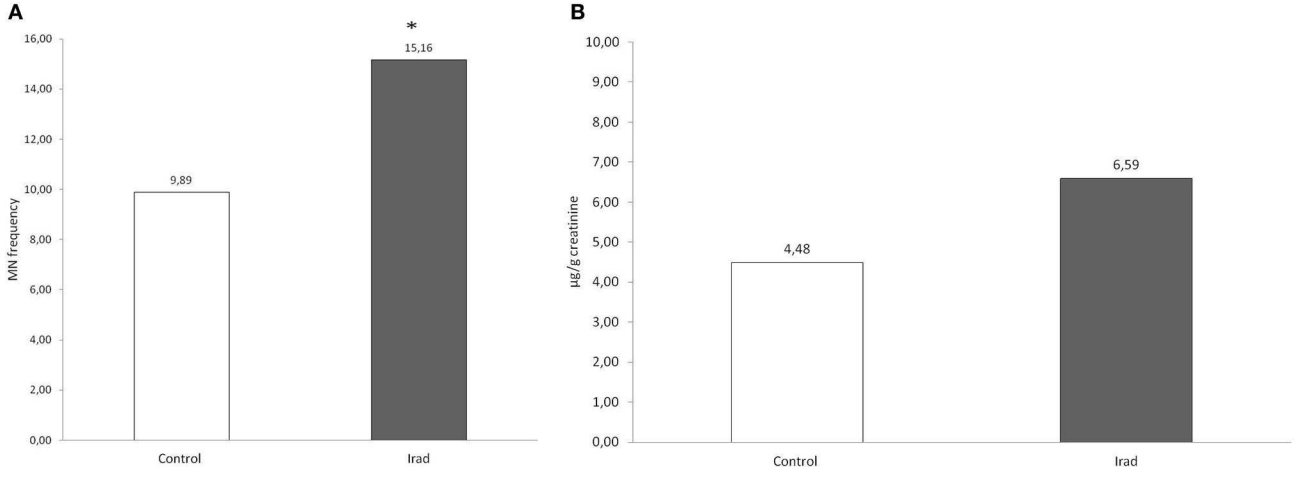

FIGURE 1 | Micronucleus frequency **(A)** and 8-OHdG values **(B)** in hospital workers exposed to ionizing irradiation. 8-OHdG, 8-hydroxy-2′-deoxyguanosine value; Control, unexposed healthy voluntairs; Irad, hospital workers exposed to Irad.

3–6 months—a group that consumed <0.5 L milk/month and a group that consumed more than 2 L milk/month. The number of subjects in each sub-group is shown in **Table 1**.

The MN frequency (**Figure 3A**) and 8-OHdG (**Figure 3B**) values in subjects from control groups who consumed more than 2 L milk/month were significantly higher ($p < 0.05$; Mann Whitney U test) in comparison to controls who consumed ≤0.5 L/month of milk. Hospital workers, occupationally exposed to ionizing radiation, who consumed more than 2 L milk/month, had also significantly higher MN frequency and 8-OHdG values ($p < 0.05$; Mann Whitney *U* test) as compared with subjects from control group.

Effect of Age, Gender, and Smoking Status on Micronuclei and 8-OHdG

The participants were divided into two sub-groups according to the age: younger (<45 years) and older (>45 years). The number of younger and older subjects in the control and exposed groups is shown in **Table 1**.

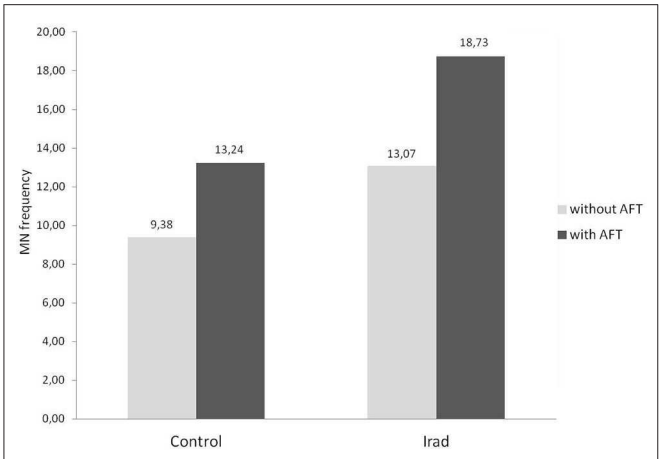

FIGURE 2 | Micronucleus frequency in relation to milk consumption in hospital workers exposed to ionizing irradiation. Control, unexposed healthy voluntairs; Irad, hospital workers exposed to Irad; *, statistically significant difference in comparison to Irad without AFT; †, statistically significant difference in comparison to control without AFT.

In all groups, older subjects had higher MN frequency compared to younger (**Figure 4A**) as well as in control group regarding to 8-OHdG values (**Figure 4B**).

As related to the gender, females had higher MN frequency than males in both control group and group exposed to Irad (**Figure 5A**) but this is not case for 8-OHdG values (**Figure 5B**).

Smokers from both groups had higher MN frequency (**Figure 6A**) and 8-OHdG values (**Figure 6B**) in comparison to non-smokers (**Table 2**).

ANCOVA showed significant differences in MN frequency ($F = 2.786$, $p = 0.043$) among the groups. The confounding factors-age ($F = 0.416$, $p = 0.520$), gender ($F = 3.021$, $p = 0.084$), and smoking status ($F = 2.222$, $p = 0.138$), were not related to the differences in MN frequency among the study groups. However, regarding to 8-OHdG values, ANCOVA showed no differences ($F = 0.393$, $p = 0.535$) among the groups. Also, the age ($F = 0.518$, $p = 0.477$), gender ($F = 0.326$, $p = 0.572$), and smoking status ($F = 0.470$, $p = 1.607$, $\eta = 0.214$), were not related to 8-OHdG levels among the groups.

DISCUSSION

This study showed that hospital workers occupationally exposed to IRad have increased values of micronuclei and 8-OHdG, which indicates DNA damage (**Figure 1**). Moreover, it can be assumed that the accidental consumption of milk in a period when AF M1 level was elevated in the majority of samples available on the market (over 86% of samples exceeded the maximum level of 0.05 mg/kg set by EU (9) contributes to an increase in values of the examined biomarkers in both control and group of subjects exposed to IRad.

It is well-known that the biological effect of the radiation is manifesting through direct and indirect DNA damage. Thereby, oxidative DNA damage by ROS exceeds the direct effect of the IRad (17). As a result of elevated ROS, transcription factors and their corresponding genes are permanently activated, which, coupled with increased DNA damage, creates the environment for the occurrence of malignant phenotype (18).

In recent years, evaluation of micronuclei became a powerful, well-accepted method in radiation biodosimetry especially for determining level of DNA damage in subjects for whom a pre-exposure analysis is not available (19) as in the case of workers exposed to IRad in our study.

The group of workers in bronchoscopy and radiotherapy units in our study had significantly higher micronucleus frequency indicating elevated genome damage due to long exposure to low doses of IRad. Also, none of the workers were accidentally irradiated, so only the cumulative effect of IRad was monitored. Results obtained in this study are in agreement with our previous study about occupational exposure to IRad (20). According to Fenech et al., increased frequency of MN is manifested by the appearance of acentric fragments and dicentric chromosomes characteristic for IRad influence, but it also provides an insight into miss-repaired DNA breaks reduced DNA reparation in our group of IRad exposed subjects (21). Radiation-induced chromosome aberrations, such as MN are the result of non-homologous end joining repair pathway, responsible for unrepaired or miss-repaired double strand breaks of DNA (22). Taking this into account, the increased MN values obtained in this study could also be the result of less efficient DNA reparation in IRad exposed group.

It is known that early biochemical changes occur immediately upon the exposure of cells to IRad. Since reactive oxygen (ROS) and nitrogen species (RNS) are continually being generated, oxidative changes happen to continue even months after the initial exposure (23). ROS and RNS, whose main generators are radiolysis of water and early activation of nitric oxide synthases under ambient oxygen can attack DNA resulting in several alterations, including DNA breaks, base damage, destruction of sugars, cross-links, and telomere dysfunction (24). The oxidized nucleosides and bases are generally excreted into urine and the base-excision repair pathway takes part in their genesis. So, based on these considerations, 8-OHdG detected in urine has been described as a sensitive marker to evaluate oxidative DNA modification (25). From a methodological standpoint, a combination of the MN with other tests, such as 8-OHdG, which measure oxidative DNA damage, can provide additional useful information.

In spite of the limitations of our study (we had restricted number of participants who consumed AFT-contaminated milk) we consider that combining indicators of DNA damage within genotoxic monitoring could give us impactful assessment of potential cumulative effect both AFT and Irad.

The group exposed to Irad had higher values of 8-OHdG in comparison to the control group, but without statistical significance. This result corresponds with MN frequency and additionally confirms that occupational IRad exposure of professional and medical staff contributes to higher DNA damage.

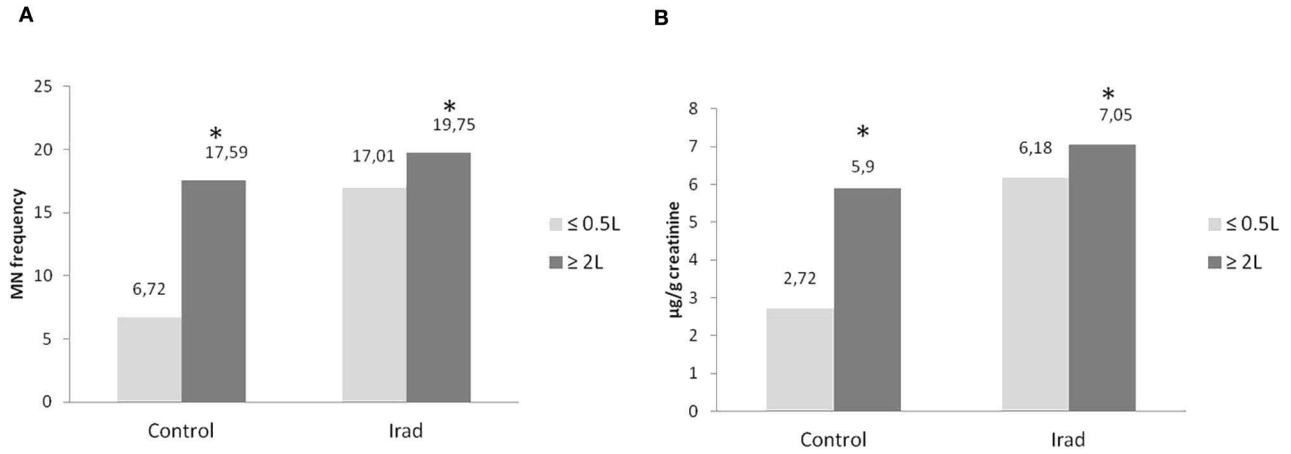

FIGURE 3 | The micronucleus frequency **(A)** and 8-OHdG values **(B)** in relation to different amounts of consumed milk with AFT in hospital workers exposed to ionizing irradiation. MN, micronucleus frequency; 8-OHdG, 8-hydroxy-2′-deoxyguanosine value; *, statistically significant difference in comparison to the control group, who consumed ≤0.5 L/month of milk.

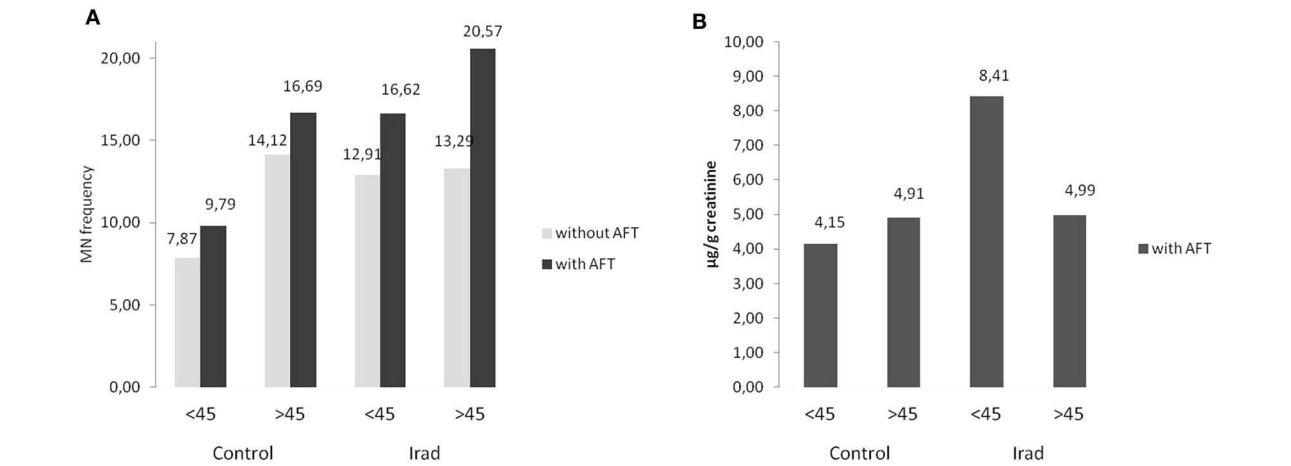

FIGURE 4 | Influence of participants' age on micronucleus frequency **(A)** and 8-OHdG values **(B)** in hospital workers exposed to ionizing irradiation. MN, micronucleus frequency; 8-OHdG, 8-hydroxy-2′-deoxyguanosine value.

Also, we presume that the absence of statistical significance for 8-OHdG values between IRad exposed and control group in our study, contrary to the significant difference obtained by MN assay, could be explained by the small number of subjects in the experimental group.

In addition, the fact that urinary 8-OHdG levels may be influenced by many factors, among which is polymorphic *hOGG1* genotype that causes inter-individual variability in 8-OH-Gua repair and has a major role in the prevention of ROS-induced carcinogenesis, should not be overlooked (15). Clearly understanding of DNA repair participant phenotypes, which exceed the purpose of this study, could give more precise answers on these questions.

During 2013, throughout collection and investigation of blood and urine samples for MN and 8-OHdG in this investigation, original literature data reported that 86% cow milk samples from Serbian market contained AF M1 at concentration higher than the approved maximum residue levels (MRL) (9). According to Jajić et al., in all 4 groups of samples from 2013 [pasteurized, raw, ultra-high temperature treated (UHT), and organic milk], very high levels of AF M1 contamination were established ranging from 80 to 100% (11).

The results of this study revealed that subjects who accidentally consumed AFT-contaminated milk had higher MN frequencies as compared to the controls (**Figure 2**). In the group of IRad exposed subjects who consumed AFT-contaminated milk, the MN frequency was significantly higher in comparison to IRad exposed ones without AFT-contaminated milk consumption. These results speak in favor that DNA damage effect is might be due to AF M1 influence.

Both of used tests confirmed that participants who consumed more AF M1 milk had more pronounced DNA damage than

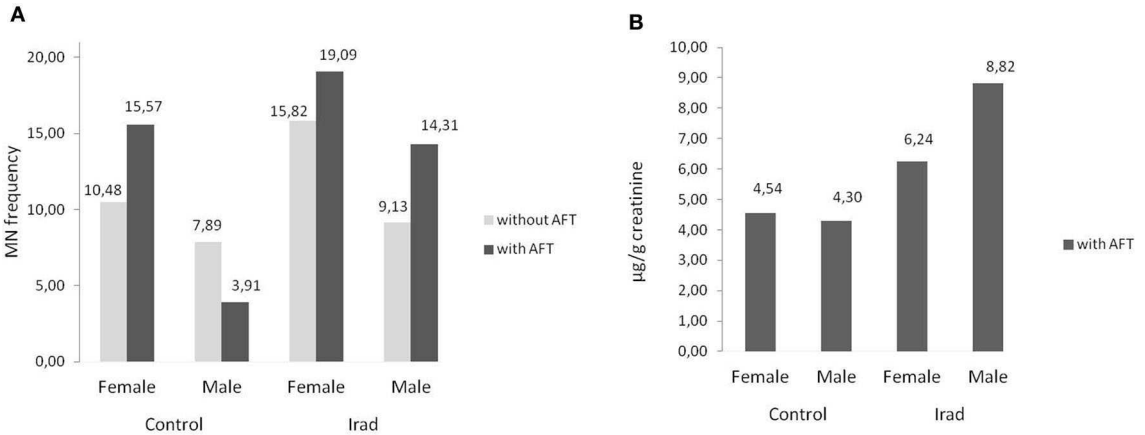

FIGURE 5 | Influence of participants' gender on micronucleus frequency. Effects on micronuclei frequencies **(A)** and on 8-OH values **(B)** in hospital workers exposed to ionizing irradiation. MN, micronucleus frequency; 8-OHdG, 8-hydroxy-2′-deoxyguanosine value.

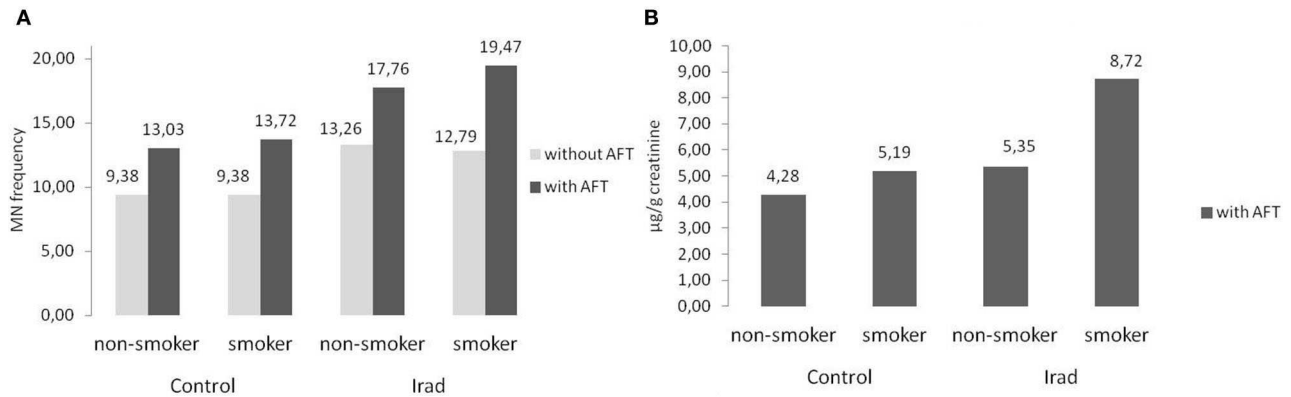

FIGURE 6 | Influence of participants' smoking status on micronucleus frequency **(A)** and 8-OHdG values **(B)** in hospital workers exposed to ionizing irradiation. MN, micronucleus frequency; 8-OHdG, 8-hydroxy-2′-deoxyguanosine value.

TABLE 2 | Multivariate analysis of the influence of age, gender, and smoking status on micronuclei and 8-OHdG.

Variables	ANCOVA (*p*-values)	
	MN frequency	8-OHdG
Irad with AFT/control without AFT	0.006*	–
Irad with AFT/Irad without AFT	0.015*	–
Control with AFT/control without AFT	0.543	–
Irad with AFT/control with AFT	0.246	0.535
Age	0.520	0.77
Gender	0.084	0.572
Smoking status	0.138	0.214

$p < 0.05$ was considered as statistically significant.

the ones who consumed less milk (**Figure 3**). The results of this study showed that consumption of higher amounts of milk in the period of AF M1 contamination corresponds with increased values of examined DNA damage biomarkers, which is in accordance with published results (5). Also previous study from 2005 which included participants who didn't consumed AFT-contaminated milk revealed lower frequency of MN in both control group and group exposed to IRad as compared to actual results (20). This difference in the degree of micronuclei frequency supports the notion about aflatoxin co-influence on DNA damage presented in this study. The cumulative effect of both AFT in milk and IRad damaging effect could be the cause of significantly increased values of MN frequency.

Concerning to confounding factors, our study showed that the effect of age is reflected in elevated MN frequency in older subjects in both control and exposed group (**Figure 4A**), which is in agreement with our previous studies (26, 27). The explanation of this phenomenon, as seen in other studies, probably lies in a combination of factors, such as cumulative effect of acquired mutations in genes involved in DNA repair chromosome segregation and cell cycle checkpoint, as well as aberrations in chromosomes caused by exposure to endogenous genotoxins and exposure to environmental or occupational genotoxins (28).

In regard to gender, the results of this study confirmed that females had higher MN frequency than males in both control group and the group exposed to Irad (**Figure 5A**). This phenomenon can be explained by a random loss of an X chromosome, which is eliminated from the nucleus to form a micronucleus (29). It reflects the importance of the gender as a variable in studies utilizing the cytokinesis-block micronucleus assay as a biomarker of chromosome damage. Similare to this results, previous studies have shown also that gender and age are not associated with changes in 8-OHdG level (30). The evaluation of influence of smoking as a confounding factor revealed that smokers in both groups had higher MN frequency and 8-OHdG values in comparison to non-smokers (**Figure 6**). Similarly, several studies showed somewhat higher 8-OHdG levels among smokers (31, 32). Since smoking causes DNA damage it has to be taken into account as a cofactor when assessing the risks of combined exposures to Irad and AF M1.

The analysis of individual confounding factors showed their slight influence on the frequency of micronuclei. However, multifactorial analysis showed that these factors were not related to DNA damage measured by 8-OHdG and micronuclei tests and also pointed out DNA damage induced by the cumulative effect of Irad and consuming AFT-milk.

CONCLUSION

This study shows increased genome damage in hospital workers occupationally exposed to low-dose ionizing radiation detected using MN and 8-OHdG assay.

Additionally, this study indicates that accidental consumption of milk with elevated aflatoxin concentrations might contribute to increased values of both investigated DNA damage biomarkers. Regardless of the limitations of our study we consider that combining indicators of DNA damage could give us impactful assessment of potential cumulative effect both AFT and Irad. It is justified to assume that co-exposure to Irad and AFT could increase health risk in occupationally exposed personnel, which point out the necessity of health risk assessment. Further investigations are required in order to mor5e closely reveal the cumulative effect of exposure to mixed radiation/chemical agents with different action mechanisms, i.e., to provide additional information about health risk of carcinogenesis in relation to eating habits.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available to protect participant identity. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute of Oncology Vojvodina, Novi Sad, Serbia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JM did the analysis and wrote the concept paper. JS approved the patients from the clinic. BS and SD did the part of the analysis. DJ helped with the analysis. VJ formulated the concept and corrected and approved the final version of the paper, as project chief. All authors contributed to the article and approved the submitted version.

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Environmental Contamination and Occupational Exposure of Algerian Hospital Workers

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Guidelines are in place to assure limited occupational exposure to cytostatic drugs. Even though this has led to a reduction in exposure, several studies reported quantifiable concentrations of these compounds in healthcare workers. In this study, we evaluated occupational exposure to cytostatic drugs in hospital workers from the University Hospital in Tlemcen, Algeria. Monitoring was performed by collecting wipe samples from surfaces, objects, personal protective equipment (gloves and masks) and from the skin of employees at an Algerian university hospital. Wipe samples were analyzed with ultra-performance liquid chromatography coupled to a mass spectrometer. Concentrations ranged from below the limit of quantification up to 208.85, 23.45, 10.49, and 22.22 ng/cm² for cyclophosphamide, ifosfamide, methotrexate and 5-fluorouracil, respectively. The highest values were observed in the oncology department. Nowadays, there are still no safe threshold limit values for occupational exposure to cytostatic agents. Therefore, contamination levels should be kept as low as reasonably achievable. Yet, healthcare workers in this hospital are still exposed to cytostatic agents, despite the numerous guidelines, and recommendations. Consequently, actions should be taken to reduce the presence of harmful agents in the work environment.

Keywords: antineoplastic drugs, chemotherapy, occupational hygiene, environmental monitoring, Africa

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INTRODUCTION

Cytostatic drugs or antineoplastic drugs are designed to damage and kill cancer cells. They are frequently used in cancer patients as chemotherapy. During preparation and administration of these harmful agents, healthcare workers can also be exposed. Exposure can occur via contact with contaminated work surfaces, equipment and patients' excreta, by manipulation of solutions containing cytostatic agents, by cleaning or by inhaling particles resulting from these actions. Dermal exposure can in turn lead to ingestion by hand-to-mouth contact (1–4). Healthcare workers are often exposed multiple times a week for several years. Since in this case cytostatic drugs only affect healthy cells, healthcare workers could potentially encounter several side effects. Mutagenic, developmental, reproductive effects and cancer were reported in the NIOSH (National Institute for Occupational Safety and Health) alert from 2004 (5). Up till now, there are still no official

exposure limits for cytostatic drugs. Some research groups formulated their own recommendations in terms of safe exposure values, e.g., Sessink (6) recommends values below 0.1 ng/cm² for cyclophosphamide. Yet, the exposure and thus the level of environmental contamination should be kept as low as reasonably achievable (5).

Multiple recommendations and guidelines have been published by among others, OSHA (Occupational Safety and Health Administration), ASHP (American Society of Hospital Pharmacists), NIOSH, ISOPP (International Society of Oncology Pharmacy Practitioners), and the Oncology Nursing Society (5, 7–10). These standards consist of recommendations on a wide range of subjects, including transport of cytostatic drugs, education and training of staff, (personal) protective equipment, monitoring of contamination, cleaning procedures, waste handling etc. Even though many recommendations and guidelines led to a decrease in occupational exposure, there is still no complete elimination of exposure (11–13). Several research groups demonstrated that even after introduction of these measures, cytostatic agents remain widespread on various surfaces and objects (3, 12–16). This can be due to non-compliancy with guidelines, limited resources or inadequacy of protocols (e.g., cleaning). Particularly in low- and middle-income countries, the limited resources can play an important role in exposure of healthcare workers and irregular environmental monitoring. Since engineering controls such as biosafety cabinets are costly, healthcare workers in low- and middle-income countries have to rely more on other measures to control hazard (e.g., personal protective equipment) (17). However, studies on safe handling of cytostatic drugs among healthcare workers in middle-income countries reported insufficient or complete lack of specialized training, inappropriate cleaning procedures and a high variability in awareness of potential hazards and use of personal protective equipment (18–23). In general, these studies indicate the need for a better implementation of guidelines. Therefore, environmental contamination and occupational exposure of healthcare workers should be monitored. Although many studies have investigated environmental exposure, not much research has been conducted using an integrative approach for different types of samples to map environmental contamination (24–27).

Our hypothesis was that even though many guidelines and recommendations are in place, there is still significant exposure of hospital workers to cytostatic drugs, especially in low- and middle-income countries. We evaluated surface contamination and the unintended occupational skin exposure to cytostatic drugs (cyclophosphamide, ifosfamide, methotrexate and 5-fluorouracil) in hospital workers from the University Hospital in Tlemcen, Algeria.

MATERIALS AND METHODS

Chemicals, Reagents, and Materials

Ifosfamide (IFO) and methotrexate (MTX) standards were European Pharmacopeia Reference Standards. Cyclophosphamide (CP) and deuterated CP (CP-d4) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). 5-fluorouracil (5-FU), 5-fluorouracil-2-13C, 15N2 (5-FU13C15N2), deuterated MTX (MTX-d3) as well as ammonium formate (AF) were purchased from Sigma-aldrich (Saint Louis, Missouri, USA). Formic acid (FA) for LC-MS was purchased from Fluka (Honeywell International Inc., New Jersey, USA). Ammonia was purchased from Chem-Lab Analytical (Zedelgem, Belgium) and UPLC-MS grade water from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol (MeOH) from J.T. Baker and HPLC-MS grade acetonitrile (ACN) were purchased from VWR International (Radnor, Pennsylvania, USA). A rotator, model reax 2 from Heidolph, Schwabach, Germany was used.

Preparation of Stock Solutions

Stock solutions of CP-d4 and 5-FU13C15N2 in MeOH with a final concentration of 100 µg/mL were made. MTX-d3 was received as a 100 µg/mL solution. These stock solutions were diluted with MeOH/ACN (8/2), resulting in three stock solutions of 10 µg/mL. Calibration stock solutions for each compound with a final concentration of 1 mg/mL in MeOH were prepared. Calibration stock solutions of 10 µg/mL were made in MeOH/water (8/2).

Analytical Procedure

Since the compounds of interest have different physicochemical properties, two separate methods were applied in order to measure the concentration of the compounds in environmental samples.

Cyclophosphamide, Ifosfamide, and Methotrexate

CP, IFO, and MTX were detected in the samples reconstituted in water/MeOH (9/1), based on a validated method, with some minor adaptations (28). Analysis was performed with an Acquity UPLC M-class system (Waters, Milford, Massachusetts, USA) and compounds were separated on a Luna Omega 1.6 µm C18 100 Å, 2.1 × 50 mm column (Phenomenex, Torrance, California, USA). Mobile phase A consisted of 0.1% FA in water and B of MeOH. Two separate gradient profiles were used (**Supplementary Table 1**). A flow rate of 0.4 mL/min and an injection volume of 10 µL were used. The outlet of the column was coupled to a Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, Massachusetts, USA) with ESI ion source. The positive ion mode was used with multiple reaction monitoring. A source temperature of 120°C and a desolvation temperature of 450°C were used together with a capillary voltage of 1.50 kV. The desolvation and cone gas flow were 800 and 25 L/h, respectively. Other MS/MS parameters can be found in **Supplementary Table 2**.

5-Fluorouracil

Analysis was performed using the method of Oriyama et al. (29), with some small modifications. An Acquity UPLC H-class PLUS system (Waters, Milford, Massachusetts, USA) was used for analysis. Samples reconstituted in ACN were injected in an Acquity UPLC BEH amide 1.7 µm, 2.1 × 50 mm column (Waters, Milford, Massachusetts, USA) for separation of 5-FU and 5-FU13C15N2. 0.01% FA in ACN was used as mobile phase

A and 10 mM AF with 0.05% ammonia in water as mobile phase B. For gradient profile see **Supplementary Table 1**. A flow rate of 0.4 ml/min and an injection volume of 10 μL were used. A Xevo TQ-XS tandem quadrupole Mass Spectrometer (Waters, Milford, Massachusetts, USA) with a UniSpray ion source was used for detection. Negative ion mode was used in multiple reaction monitoring. An impactor voltage of 0.50 kV and desolvation temperature of 450°C were applied. A desolvation, cone and nebuliser gas flow of 600, 150 L/h, and 7.0 bar were used, respectively (**Supplementary Table 2**).

Method Validation

An extraction solvent containing 20 ng/mL of internal standards CP-d4, MTX-d3 and 5-FU13C15N2 was prepared by mixing each internal standard stock solution with MeOH/ACN (8/2). The first calibration working solution with final concentrations of 1,000 ng/mL containing CP, IFO, MTX, and 5-FU was made by mixing each calibration stock solution together in MeOH/water (8/2). The second calibration working solution was obtained by diluting the first calibration working solution with MeOH/water (8/2) to result in a final concentration of 50 ng/mL. These two calibrations working solutions were used to spike TX714K low TOC Alpha Swab Series of 100% polyester (Texwipe, Kernersville, North Carolina, USA) with increasing concentrations of cytostatic drugs (0, 1, 5, 10, 30, 70, 100, 300 ng/swab). This was done in triplicate. After spiking, the swabs for calibration were each placed in separate glass vials and snapped at the notch of the handle. Ten mL of extraction solvent containing 20 ng/mL of internal standards, was added to the glass vials containing the swabs. The vials were shaken and rotated for 30 min. Subsequently, two times 4.5 mL was transferred to separate test tubes and evaporated under a nitrogen gas stream. The dry samples were then reconstituted in 900 μL MeOH/ACN (8/2), vortexed and transferred to injection vials. These vials were again dried out and half of them was reconstituted in 300 μL water/MeOH (9/1), while the other half was reconstituted in 300 μL ACN. After vortexing, the samples were injected. The limit of quantification (LOQ) was defined as the lowest concentration for which the precision was below 20% and the accuracy between 80 and 120%. The accuracy was calculated as the average of the estimated concentration divided by the nominal concentration, multiplied by 100 and the precision as the standard deviation of the estimated concentration divided by the average of the estimated concentration, multiplied by 100.

Field Study: Evaluation of Surface Contamination and Occupational Exposure

In a university hospital in Algeria, samples were collected in 6 different departments. More specifically, surfaces, objects, personal protective equipment (PPE), and the skin of healthcare workers were sampled in the dermatology, maternity oncology, oncology, hematology, nephrology, and rehabilitation departments.

Sample Collection

Cytostatic drug sampling kits containing swabs, a square template with a 10 × 10 cm opening and a vial containing MeOH/water (8/2) were provided by the Laboratory for Occupational and Environmental Hygiene (LOEH, Leuven, Belgium) for surface sampling. Surface samples were collected using TX714K Low TOC Alpha Swab Series (Texwipe, Kernersville, North Carolina, USA). After the swab was immersed in MeOH/water (8/2), it was pushed against the walls of the vial and wiped across the rim to remove air and expel any excess of solvent. This is important to avoid inconsistent results. The swab was then used to wipe a surface using the provided template and according to a specific pattern. The first side of the first swab was wiped horizontally across the opening of the template, the second side was used to wipe the same area vertically. A second swab was used if the surface area was over 100 cm^2 and when possible, the area was measured. The second swab was used to wipe the area diagonally upwards with one side and diagonally downwards with the other side. After sampling, both swabs were placed in the glass vial containing the remaining solvent. The vial was then closed and stored at −20°C until shipment to Belgium. Dermal samples and samples from objects were taken by use of the same sampling kit. PPE sampling involved wiping the front and back side of gloves and facial masks using two swabs and was performed in the Laboratory for Occupational and Environmental Hygiene (Leuven, Belgium). All samples were stored at −80°C until use.

Sample Extraction

Ten mL of extraction solvent was added to the glass vials containing the swabs. From this point forward, exact the same process was followed as for the calibration samples.

TABLE 1 | Method validation parameters.

Compound	Accuracy (%)	Precision (%)	R^2	LOQ (ng/sample)
CP[a]	109.97	4.31	0.9959	10
IFO[a]	80.54	7.52	0.9989	30
MTX[a]	105.26	6.34	0.9968	5
5-FU[a]	95.76	4.59	0.9962	10

[a]*CP, cyclophosphamide; IFO, ifosfamide; MTX, methotrexate; 5-FU, 5-fluorouracil.*

RESULTS

Method Validation

Calibration curves were based on eight concentration levels (0, 1, 5, 10, 30, 70, 100, and 300 ng/swab). Curves for all compounds had correlation coefficients R^2 exceeding 0.99. The limits of quantification (LOQs) were based on the definition mentioned in the materials and methods section and were 10 ng/sample for CP, 30 ng/sample for IFO, 5 ng/sample for MTX and 10 ng/sample for 5-FU. More detailed information on accuracy, precision and linearity is summarized in **Table 1**.

Field Study: Evaluation of Surface Contamination and Occupational Exposure

In total 62 samples were collected in 6 different departments in the University Hospital. These samples included 39 surface samples, 10 samples from PPE and 13 dermal samples. Surface samples were collected from various areas and objects such as door handles, tables, hoods, a calculator, a telephone, sinks, chairs… Samples from PPE were taken from gloves and facial masks. Face, hands and arms were swabbed to collect dermal samples. In general, the highest concentrations were observed in surface samples, followed by samples from PPE, and dermal samples (**Figure 1**).

Surface Samples

In case surface areas were measured, concentrations were recalculated to ng/cm² to present the results. All surface samples tested positive on one or more cytostatic compounds. More than half of the 39 analyzed samples tested positive for CP (79.5%), MTX (56.4%), and 5-FU (66.7%). IFO was above the LOQ in 30.8% of the samples. CP was not only found in the highest number of positive samples, but also in the highest measured concentration per cm² (208.85 ng/cm²) (**Figure 1A**). The maximum surface contaminations for IFO, CP and 5-FU were found on a calculator in the oncology department (23.45, 208.85, and 22.22 ng/cm², respectively) The highest concentration of MTX was found on a work schedule in the same department (10.49 ng/cm²). In the oncology department all but one sample tested positive for MTX. Also, substantial concentrations of CP, IFO, and 5-FU were measured in samples from the hood before and after preparation, the telephone, the water tap after preparation and the door handle. All surface samples in the rehabilitation department tested positive for 5-FU. IFO and MTX were detected in low concentrations on a table in the treatment room (0.00–0.01 ng/cm²) and a non-identified sample (253.6 ng/swab IFO, 66.4 ng/swab MTX). CP could be found on a door handle (0.22 ng/cm²) and the same unidentified sample (639.1 ng/swab). Only one surface sample from the nephrology department was provided. This sample, taken from the preparation table, contained a high concentration of CP (25.25 ng/cm²) and only a low concentration of 5-FU (0.01 ng/cm²). The concentrations of IFO and MTX were below the LOQ. All but one sample collected in the hematology department tested positive for CP. Low concentrations of IFO, MTX and 5-FU were observed in samples from a drawer (0.03 ng/cm² IFO), fridge (0.03 ng/cm² IFO, 0.01 ng/cm² MTX and 5-FU), tap (0.54 ng/cm² MTX, 0.07 ng/cm² 5-FU), and sink (0.04 ng/swab MTX). Except for one sample from a chair (0.03 ng/cm² IFO), all samples in the maternity oncology department had IFO concentrations below the LOQ. CP was observed to be above the LOQ in all surface samples. Especially, a sample from the hood after use contained a high concentration of CP (13.84 ng/cm²). MTX was observed in samples of the sink, tap and a non-identified sample. 5-FU was detected in quantifiable concentrations on the hood after use (1.70 ng/cm²) and on the same chair (0.06 ng/cm²), sink (0.21 and 0.02 ng/cm²), tap (6.07 ng/cm²), and non-identified sample. In the dermatology department, all surface samples tested positive for CP. IFO could only be quantified on a door handle (1.28 ng/cm²), MTX on a door handle (4.01 ng/cm²) and a preparation table (0.001 ng/cm²) and 5-FU on a door handle (1.71 ng/cm²) and in a sample of the preparation table (0.002 ng/cm²). More detailed information can be found in **Supplementary Table 3**.

Samples of PPE

The concentrations found on masks were recalculated to ng/cm² based on the total surface area (315 cm²). For the gloves, a total surface area of 800 cm² was used to recalculate, consistent with the surface area of hands according to the World Health Organization (30). Seven out of 10 analyzed samples contained at least one cytostatic compound. Four samples contained CP in concentrations that exceeded the LOQ, one sample for IFO, three for MTX, and three for 5-FU. The highest concentration was observed for MTX (2.66 ng/cm²) (**Figure 1B**). IFO (0.18 ng/cm²) was only quantifiable in gloves used for preparation by a nurse working in the oncology department. CP was quantified on gloves from a nursing aide in the nephrology department (0.50 ng/cm²) and gloves (0.79 ng/cm²) and two masks (0.03 and 0.11 ng/cm²) from nurses in the oncology department. Substantial concentrations of MTX (2.66 ng/cm²) were detected on gloves from the rehabilitation department, whereas low concentrations were seen in samples of gloves from the maternity oncology (0.01 ng/cm²) and dermatology (0.01 ng/cm²) departments. Low concentrations of 5-FU were found on the gloves of a nurse from the nephrology department (0.01 ng/cm²) and a mask from the oncology department (0.34 ng/cm²), while a higher concentration of 2.15 ng/cm² was detected on the gloves used for the preparation of cytostatic drugs in the oncology department. For further information, consult **Supplementary Table 3**.

Dermal Samples

Also for the samples collected from the skin, recalculations were performed according to surface areas mentioned by the World Health Organization (30). Surface areas of 800 cm² for hands, 1,200 cm² for arms and 650 cm² for the face were used. Eight out of 13 analyzed dermal samples tested positive for one or more cytostatic compounds. CP was found in quantifiable concentrations in 7 out of 13 samples. IFO was only present in one sample, 5-FU in five samples and MTX in none of the tested samples. The highest concentration was measured on the face of a nurse active in the oncology department (0.28 ng/cm² IFO) (**Figure 1C**). None of the samples contained MTX concentrations above the LOQ. CP was present in only low concentrations on the face and arms of the same nurse from the oncology department (0.11 ng/cm² on face and 0.03 ng/cm² on arms), the faces of two nurses in the hematology department (0.02 ng/cm²) and body samples from the nurse and psychologist from the maternity oncology department (0.03 and 0.02 ng/cm²). For 5-FU low concentrations were detected in samples from the faces of nurses in the nephrology (0.03 ng/cm²) and oncology (0.05 ng/cm²) departments, arms of a nurse in the oncology department (0.03 ng/cm²), body samples of a psychologist (0.01 ng/cm²) and hands of a nurse from the rehabilitation department (0.02 ng/cm²). Further information is listed in **Supplementary Table 3**.

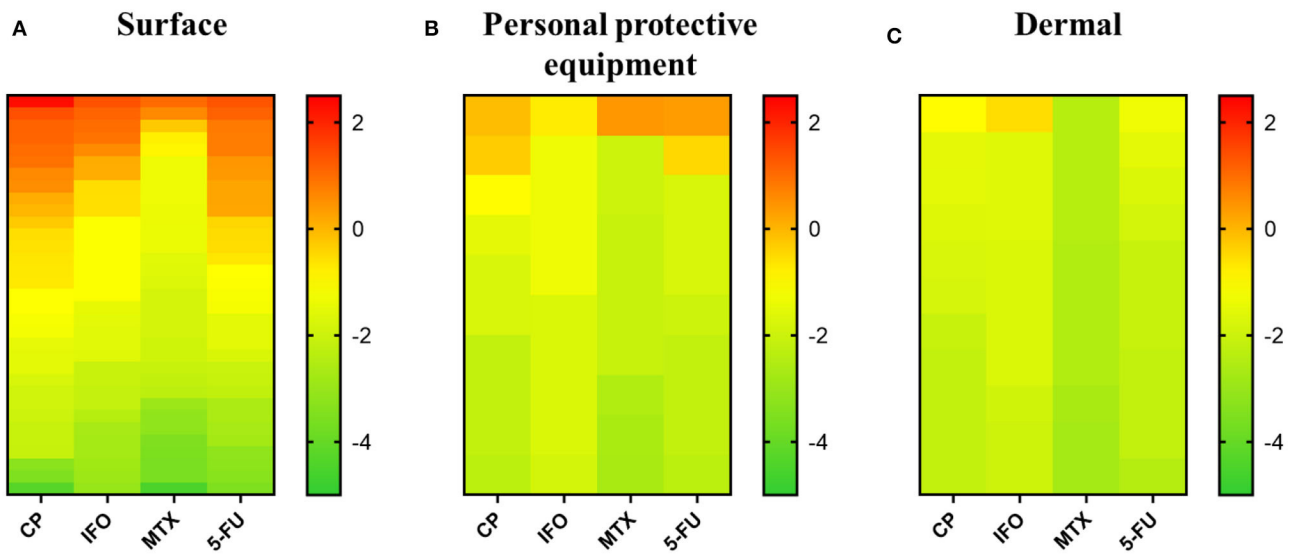

FIGURE 1 | Overview of cytostatic drug concentration in surface, personal protective equipment and dermal samples from the university hospital, Algeria. All three panels give the logarithmic scale of cytostatic concentrations (ng/cm²) ranked from high to low for each cytostatic drug. **(A)** Cytostatic concentrations found in surface samples. **(B)** Cytostatic concentrations found in samples of personal protective equipment. **(C)** Cytostatic concentrations in dermal samples. CP, cyclophosphamide; IFO, ifosfamide; MTX, methotrexate; 5-FU, 5-fluorouracil.

DISCUSSION

The aim of this study was to evaluate the environmental cytostatic contamination and unintended skin exposure in healthcare workers of an Algerian university hospital, by means of surface sampling. Absorption through the skin is the main route of occupational exposure for cytostatic agents (31). This implicates that surface contamination of hazardous substances possess an important exposure risk. As expected, the highest surface concentrations of cytostatic compounds were observed in the oncology department while the lowest concentrations were found in the rehabilitation and nephrology departments. This is in line with what was expected, since cytostatic drugs are most often used in the oncology department as part of cancer therapy. Out of the 33 samples with known surface area, 11 had a concentration below 0.1 ng/cm² for all cytostatic drugs. These samples were collected in the rehabilitation, oncology, hematology, maternity oncology and dermatology department. In 14 samples, levels from 0.1 up to 10 ng/cm² were detected. Low surface contamination indicates that healthcare workers were handling cytostatic drugs with care and that the cleaning procedures were adequately performed. Nonetheless, this can also be due to less frequent use of cytostatics in these departments. Low levels can still lead to exposure of healthcare workers when touched with bare hands (e.g., door handle). Even though most of the samples had concentrations below 10 ng/cm², 8 out of 33 samples contained extremely high concentrations of cytostatic drugs. Six of these samples were from the oncology department. In this department, samples from the hood contained 10.21 ng/cm² CP (before preparation) and 14.06 ng/cm² 5-FU (after preparation). As cytostatic drugs are most often handled inside biosafety cabinets, these high concentrations were expected. The high concentrations before use of the hood do indicate poor cleaning procedures. On the calculator, phone, work schedule, and door handle of the oncology department, concentrations exceeding 10 ng/cm² were found. The surface samples from the calculator contained even higher concentrations of several cytostatic compounds (208.85 ng/cm² CP, 23.45 ng/cm² IFO, 22.22 ng/cm² 5-FU) than observed on the hood after preparation of these compounds. This is probably due to spillage or secondary transfer by contact in combination with inadequate cleaning. Contamination of the telephone and door handle can form a risk for the hospital personnel, since these are often handled with bare hands. In the nephrology and maternity oncology department very high concentrations of CP were observed in samples from a preparation table (25.25 ng/cm²) and the hood after preparation (13.84 ng/cm²). Such high concentrations only occur after spilling and the lack of adequate cleaning, while low surface concentrations are more an indication of careless working. High surface contamination results in a high risk for personnel to get exposed, with even the possibility to transfer this contamination to third parties. If we compare our range of surface CP contamination (< LOQ−208.85 ng/cm²), with the literature, which reported concentrations from < LOQ to 14 ng/cm², substantially higher contamination was observed in our study (12, 25, 27, 32–35). Müller-Ramírez et al. (14) reported values ranging from 0.3 to 168.9 ng/cm², which is more in line with our results, while only one study reported ranges exceeding ours with contamination up to 21,300 ng/cm² (36). This concentration was found on a phone in a room next to the preparation room and was probably caused by touching the phone with contaminated gloves and inadequate cleaning.

For IFO we observed concentrations ranging from < LOQ up to 23.45 ng/cm², which is in line with concentrations (0.08–15.7 ng/cm²) reported by Müller-Ramírez et al. (14). Other studies reported substantially lower contamination (12, 25, 33, 35), or considerably higher contamination (< LOQ−95 ng/cm²) (32, 37). Hedmer and Wohlfart (32) described one really high concentration of 95 ng/cm² on the floor of a patient lavatory, which is probably coming from the patients' excreta. In our study MTX concentrations ranged from < LOQ to 10.49 ng/cm². Also for MTX surface contamination, low to very high concentrations are reported in literature, ranging from 0.515 to 51 ng/cm² (12, 33, 37). Finally, in our study surface 5-FU concentrations ranged from below the LOQ to 22.22 ng/cm², which is similar to Kiffmeyer et al. (37). Lower ranges were found by Koller et al. (27) and Kopp et al. (33) from < 0.007 up to 14.56 ng/cm². Schierl et al. (38) and Viegas et al. (36) reported higher ranges (< LOQ−253.33 ng/cm²). Interpretation of the level of contamination is difficult, since there are no official threshold limit values. Yet, several research groups have already described this problem in different ways and have proposed own "in-house" thresholds. Schierl et al. (38) used the median and 75th percentile of all observed concentrations in surface samples to define two threshold values, namely concentrations below the median indicate good working practices, while values above the 75th percentile indicate the need for optimization of the handling procedures. For 5-FU these thresholds are 0.03 ng/cm² for the lower limit and 0.005 ng/cm² for the upper limit. Likewise, other research groups used the 90th percentile as guidance value. Kiffmeyer et al. (37) established a guidance value of 0.1 ng/cm², independent of the substance measured. This value was based on the highest concentration of all the compounds that were quantified. Different guidance values for different sampling areas and rooms where cytostatic drugs are handled were proposed by Hedmer and Wohlfart (32). Sottani et al. (39) used the 90th percentile to propose guidance values for CP (3.6 ng/cm²), 5-FU (1.0 ng/cm²), gemcitabine (0.9 ng/cm²), and platinum (0.5 ng/cm²). Furthermore, Sessink (6) coupled guidance values to actions to be taken to ensure a safe environment. CP is commonly used in chemotherapy. It can permeate the skin easily and is extremely toxic. Therefore, determination of guidance values was based on the 90th and 99th percentile of CP concentrations in wipe samples. The 90th and 99th percentile correspond to 0.1 and 10 ng/cm², respectively. Surface concentrations below 0.1 ng/cm² are presumed to be safe, while levels above 10 ng/cm² are considered to be unacceptable. Depending on the concentration found, different actions should be performed. For concentrations lower than 0.1 ng/cm², the environment should be monitored once a year and evaluated after 4 years. Surfaces containing 0.1–10 ng/cm² require risk estimation, monitoring within 3–6 months and action taking if necessary. For concentrations exceeding 10 ng/cm², taking action and follow-up of these improvements is highly recommended. Applying these thresholds to our own data, we observed that 15 out of 33 surface samples had concentrations between 0.1 and 10 ng/cm² for at least one compound, whereas 8 contained more than 10 ng/cm² of one or more cytostatic drugs. In other words, ∼70% of the surface samples contained levels exceeding the safe level according to Sessink (6). This points at an urgent need for action. Since it can be difficult to reduce exposure by use of costly engineering controls in middle-income countries, such as Algeria, the focus should be on administrative controls as well as personal protective equipment. Administrative controls include education and training, safe handling policies and medical surveillance. Education and training can raise awareness of hospital personnel on the potential hazards of working with cytostatic agents. Previous research in low- and middle-income countries showed that this could be a reason for non-compliancy with safety guidelines (18–23). Some extremely high levels of cytostatics were probably caused by spilling and remained on the surfaces by inadequate cleaning or cleaning protocols. This source of exposure can be reduced by adequate safe handling policies and procedures and by provision of proper equipment (e.g., biosafety cabinets, PPE, closed-system drug transfer device). Our results suggest that the current cleaning procedures should be checked by regularly performing measurements before and after cleaning. If the existing cleaning protocols then seem inadequate, changes need to be made to the protocol, and monitoring should show improvement. Since there was a lack of functioning biosafety cabinets and PPE, urgent investments are necessary. This should include spill kits and PPE in areas where harmful agents are handled, along with preferably closed-system drug transfer devices. Sessink et al. (40) found a substantially lower environmental contamination in hospitals after using the closed-system drug transfer devices compared to using standard drug preparation techniques. Finally, in this case, medical surveillance and environmental surveillance is highly recommended due to the high concentrations found on several surfaces and objects.

Next to surface sampling, we collected samples from PPE. Here, we also found the highest concentrations of CP, IFO, and 5-FU in the oncology department. MTX was found in the highest concentration in the rehabilitation department. In the other departments, low concentrations were measured. In literature, CP concentrations up to 0.68 ng/cm² on gloves were reported, which is in line with our findings (< LOQ−0.79 ng/cm²) (24, 26, 27, 41). For IFO concentrations ranging between 0.14 and 2.26 ng/cm² are described, whereas our results (0.18 ng/cm²) are at the lower end of this range (24, 26). On the other hand, we found MTX concentrations up to 2.66 ng/cm², while another study showed much lower concentrations on PPE (below 0.1 ng/cm²) (24). For 5-FU, we found a maximum concentration of 2.15 ng/cm² on gloves. While some authors reported values well below this concentration (0.018–0.33 ng/cm²), others reported concentrations high above these values (449.3 and 11.4 ng/cm²) (26, 27, 36, 41). In total, 10 samples were analyzed from PPE (masks and gloves). Six out of these samples had concentrations below 0.1 ng/cm² for all compounds of interest, whereas the remaining 4 samples all had concentrations below 10 ng/cm². Even though there were no extremely high concentrations detected, 7 out of 10 samples contained quantifiable concentrations of at least one cytostatic compound. This can be caused by spilling, transfer from contaminated surfaces and objects (e.g., vials) to gloves and masks. The most important safety measures that should be taken

in terms of PPE are first of all the unconditional use of PPE and the regularly changing of gloves, mask and lab gown, especially after spills.

In total, 13 dermal samples with known surface area were analyzed. None of these samples contained high concentrations of cytostatic drugs. Few studies have been measuring dermal exposure to cytostatic drugs by use of the wiping technique (42–45). Hon et al. (42, 43) reported CP concentrations up to 0.028 ng/cm² on the skin, while in this study higher concentrations up to 0.11 ng/cm² were observed. Hon et al. (42) also reported low dermal MTX contamination ranging from < LOQ to 0.0003 ng/cm². Although there was substantial MTX in the surface samples, we found no MTX on the skin (LOQ < 5 ng/sample). Five out of 13 samples had concentrations below the LOQ for all 5 compounds, 7 had concentrations between 0.01 and 0.03 ng/cm² and only 1 had a concentration exceeding 0.1 ng/cm². This exposure can be caused by touching the face with contaminated gloves. Therefore, it is important to wear PPE and to avoid contact with bare skin. Bos et al. (46) proposed a dermal occupational exposure limit of 4 ng/cm² for CP. There were some limitations in our study. A first one is that not all vials were packed well for shipment, resulting in partial loss of some samples and turning some identification labels unreadable. Also, not all surface areas were measured because of their complex shape. Due to the unreadable labels and missing areas of some surfaces, the results of 6 samples could not be compared to other samples (in ng/cm²) or interpreted. We also only had one surface sample from the nephrology department, which makes it difficult to get a good view of this department. For PPE and dermal samples we only had a limited number of samples. Therefore, it is difficult to estimate the actual exposure of the health care workers in these departments. We also have to take into account that the sampled area is not necessarily representative for the total surface of the object (e.g., hood, table).

In the future, this environmental monitoring needs to be complemented by biological monitoring. A combination of both approaches will give a more profound insight in the actual occupational exposure of healthcare workers.

CONCLUSION

Despite the numerous guidelines and recommendations, there is still a significant exposure of healthcare workers to cytostatic drugs in the hospital examined. This study offers a first perspective on the occupational exposure in an Algerian healthcare setting and shows that significant concentrations of hazardous compounds are present on a broad range of surfaces and objects in different departments of the hospital. These results can be interpreted as a call for action. Regular environmental and biological monitoring can give a clear idea about the corrective actions to be taken in the future and will further enable follow-up of improvements.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

EV, MC, JV, MZ, TA, RL, KP, R-CD, and LG: conception and design. EV and MC: analysis. EV, MC, LG, R-CD, KP, and JV: interpretation. EV and MC: drafting manuscript. LG, R-CD, KP, JV, RL, MZ, and TA: proof reading. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00374/full#supplementary-material

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Direct and Oxidative DNA Damage in a Group of Painters Exposed to VOCs: Dose – Response Relationship

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Volatile organic compounds (VOCs) are present in several working activities. This work is aimed at comparing oxidative stress and DNA damage biomarkers to specific VOCs in the occupational exposure of painters. Dose-response relationships between biomarkers of oxidative stress and of dose were studied. Unmetabolized VOCs and their urinary metabolites were analyzed. Urinary Methylhyppuric acids (MHIPPs, xylenes metabolite), Phenylglyoxylic and Mandelic acid (PGA, MA ethylbenzene metabolites), S-Benzylmercapturic acid (SBMA, toluene metabolite), and S-Phenylmercapturic acid (SPMA, benzene metabolite) were quantified at the end of work-shift. Oxidative stress was determined by: urinary excretion of 8-oxodGuo, 8-oxoGua and 8-oxoGuo and direct/oxidative DNA damage in blood by Fpg-Comet assay. Multivariate linear regression models were used to assess statistical significance of the association between dose and effect biomarkers. The regressions were studied with and without the effect of *hOGG1* and *XRCC1* gene polymorphisms. Statistically significant associations were found between MHIPPs and both 8-oxoGuo and oxidative DNA damage effect biomarkers measured with the Comet assay. Oxidative DNA damage results significantly associated with airborne xylenes and toluene, whilst 8-oxodGuo was significantly related to urinary xylenes and toluene. Direct DNA damage was significantly associated to SBMA. *XRCC1* wild-type gene polymorphism was significantly associated with lower oxidative and total DNA damage with respect to heterozygous and mutant genotypes. The interpretation of the results requires some caution, as the different VOCs are all simultaneously present in the mixture and correlated among them.

Keywords: biological monitoring, genotoxicity, oxidative stress, urinary dose biomarkers, gene polymorphism, volatile organic compounds

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INTRODUCTION

Volatile Organic Compounds (VOCs) include a variety of chemicals present in many household products and used in several working activities. It is widely known that continuous exposure to these compounds is dangerous, as these substances have been classified as carcinogenic by IARC (1) with adverse effects on the human health. Because of the easy evaporation at room temperature, the

organic solvents can spread easily in the environment and are toxic. Particularly in the occupational setting, VOCs must be handled following appropriate safety precautions such as wearing the personal protection equipment (PPE) in order to avoid excessive exposure.

A bibliometric analysis, published in 2019, examined the scientific literature published in the years 2016–2018 on VOCs' effect on human health. The investigators conclude that the most common diseases, potentially associated with VOCs, mainly involve the respiratory system, the blood system, and inflammation (2).

One of the main effects induced by VOCs on humans is the damage of nucleic acids causing oxidative stress, genotoxicity and inflammation (3, 4). Nucleic acid oxidative damage biomarkers are the first biomarkers indicating a potential risk of chronic diseases, including chronic obstructive pulmonary disease (COPD), as well as lung, bladder cancer and hematological malignancies (5).

The human biological monitoring of exposure consists in the determination of biomarkers, which can be dose biomarkers, measuring internal exposure levels to be compared with any (if known) biological limit value, effect biomarkers, which highlight early symptoms or dysfunctional situations still reversible with the improvement of the exposure situations, and susceptibility biomarkers, which express individual differences of genetic or acquired origin (6).

The biomonitoring of workers exposed to VOCs can provide a useful and early detection system for the initiation of cell dysregulation, which would help to prevent the development of disease (7).

Painters represent an important worker category exposed to VOCs and several studies investigating the potential effects of exposure to toluene, xylenes, ethylbenzene, styrene and paints in this worker population showed oxidative and genotoxic consequences (8–13). In particular, the study of Moro et al. (11) found induction of DNA damage evaluated by comet assay, but not of micronuclei, in industrial painters exposed to low toluene levels. An increase of oxidative DNA injury in occupational exposure to paint was reported by Chang et al. (14). The authors found in spray painters a significant correlation between urinary 8-hydroxydeoxyguanosine (8-OHdG) and exposure to ethylbenzene. The recent study of Londono-Velasco et al. (9) found that exposure of car painters to organic solvents and paints was associated to an increase of the oxidative damage to the DNA evaluated by Fpg comet assay on lymphocytes.

In the present study we analyzed in a group of 17 painters the exposure biomarkers indicating the oxidative stress on single base/nucleotide (8-oxoGuo, 8-oxodGuo, and 8-oxoGua) (15, 16) as well as the effect biomarkers as direct and oxidative DNA damage (17, 18). Since the metabolism of such compounds is carried out by specific enzymes, we took into account also the role of susceptibility biomarkers that were analyzed by genotyping (19). One is *hOGG1*, i.e., the human *hOGG1* gene encoding the 8-oxoguanine DNA glycosylase, whose activity is to catalyze the excision of the mutagenic lesion 7,8-dihydro-8-oxoguanine (8-oxoGua) from oxidatively damaged DNA. Sub-cellular location of this protein is in the nucleus and in the mitochondrion (https://www.uniprot.org/uniprot/O15527) (20). The other gene is *XRCC1* (X-ray repair cross-complementing protein 1), an enzyme involved in DNA single-strand break repair by mediating the assembly of DNA break repair protein complexes. *XRCC1* is an essential protein required for the maintenance of genomic stability as it is involved in DNA repair system. The main function of *XRCC1* is associated with its role in the single-strand break (SSB) and base excision repair (BER) pathways that share several enzymatic steps. *XRCC1* has a crucial role in the coordination of BER pathway and its interaction with *OGG1*, in modulating DNA reparative response, is reported (21). Both enzymes are responsible for the maintenance of the DNA integrity (22).

The objective of this work is to evaluate the association between dose and effect biomarkers, related to both oxidative stress and damage of nucleic acids in the exposure to relatively low dose of VOCs. In particular, the urinary concentrations of Methylhyppuric acids (MHIPPs, xylenes metabolite), Phenylglyoxylic and Mandelic acid (PGA, MA both ethylbenzene metabolites), S-Benzylmercapturic acid (SBMA, toluene metabolite) and S-Phenylmercapturic acid (SPMA, benzene metabolite) were quantified at the end of the work-shift. The oxidative stress was determined by means of different effect biomarkers, such as the direct oxidation products generated from the DNA and RNA repair and turnover as well as direct and oxidative DNA damage evaluated by Comet assay, with and without Fpg.

Multivariate linear regression models were used to assess the statistical significance of the association between dose and effect biomarkers. The regressions were studied with and without the effect of the *hOGG1* and *XRCC1* gene polymorphisms to find out the most susceptible genotypes in the repair of DNA damage. A major limitation of this study is related to the fact that several different VOCs are simultaneously present in the mixture. In order to evaluate how the different effect biomarkers are sensitive and specific with respect to each VOC, subjects exposure to each VOC should have been separately available. This ideal circumstance is typical of animal studies, and almost never occurs in occupational health field studies, especially when the low dose regime is what one is interested in. Keeping in mind its limitation, this study could prompt and represent a basis for further field studies with different exposure conditions and for controlled exposure animal studies.

MATERIALS AND METHODS

Subjects and Study Design

For this work were enrolled 17 professional painters working in a naval industry in Central Italy.

During the same experimental campaign, other data were collected on the same workers. In particular audiological data,

Abbreviations: VOCs, Volatile organic compounds; OELs, Occupational Exposure Limits; BEI, Biological Exposure Index; SPMA, S-Phenylmercapturic acid; SBMA, S-Benzylmercapturic acid; MHIPPs, Methylhippuric acids; PGA, Phenylglyoxylic acid; MA, Mandelic acid.

showing the hearing dysfunctionality in workers exposed to VOCs (23), and miRNA data were separately analyzed in other studies (24). The present study was formally approved by the local Ethic Committee of the Health local agency of the Region of Marche. The workers gave their informed consent in participating to the study. Two main working tasks have been identified, roller- and spray-painting, the latter being associated to a larger airborne concentration of aromatic solvents of the mixture. The workers used respirators with carbon filters as the potential exposure levels to VOCs are of the order of the Occupational Exposure Limits of the Italian legislation. All the subjects were male, two of them belonging to Caucasian ethnicity and the others to Bengalese ethnicity. The mean age was 39 years (range 21–54 years). Five workers were smokers. The exposure to solvents was assessed by personal air sampling and urine sampling performed before and after the work-shift. An anamnestic questionnaire was administered to the enrolled subjects under the researchers supervision, regarding the professional exposure to organic solvents but also about the personal lifestyle and habits, the general health status, the cigarette smoke and use of drugs. The experimental campaign was performed on June 25th, 2018.

Personal Air Monitoring

The personal exposure to organic solvents was assessed by passive air sampling by means of Radiello® devices during the whole work-shift. Each Radiello was chemically extracted with carbon disulfide and the samples were analyzed by GC-MS (G1888A, coupled with a 6890N, AgilentTechnologies, Santa Clara, CA United States) equipped with a single quadrupole mass spectrometric detector (5973 MSD System, Agilent Technologies) with the internal standard method for the target VOCs, namely ethyl acetate, benzene, toluene, ethylbenzene, p-xylene, m-xylene, o-xylene.

Biological Monitoring

Unmetabolized VOCs in the urine were determined by GC-MS with the headspace analysis method (25). The concentrations of toluene, ethylbenzene, p-xylene, m-xylene, o-xylene excreted unchanged were measured in the urine samples at the beginning and the end of the work-shift. In the same samples, for each VOC, the concentration of its most common and specific urinary metabolite was also determined. These are 2, 3, and 4-methylhippuric acid (MHIPPs, metabolites of 2, 3, and 4-xylene), Phenylglyoxylic and Mandelic acid (PGA, MA both ethylbenzene metabolites), S-Benzylmercapturic acid (SBMA, toluene metabolite) and S-Phenylmercapturic acid (SPMA, benzene metabolite). The cotinine, the most specific metabolite of nicotine, as measured at the aim of quantifying the exposure to cigarette smoke.

All the metabolites have been determined by HPLC-MS/MS (Series 200 LC pump, PerkinElmer, Norwalk, CT, USA coupled with an API 4000 triple-quadrupole mass spectrometry detector, AB/Sciex, Ontario, Canada) equipped with a Turbo Ion Spray (TIS), in the urine samples of workers, both before and after the working shift. SPMA, cotinine, their deuterium-labeled internal standards and MA and PGA were determined following the method described in Tranfo et al. (26), and in Paci et al. (27), respectively. SBMA and MHIPPs were determined by suitably changing the method by Sabatini et al. (28).

The 8-oxoGua 8-oxodGuo, 8-oxoGuo and their internal standards were determined by following the method described in Andreoli et al. (29) with some modifications (15).

All the results were expressed as the ratio to the concentration of urinary creatinine, in order to normalize the results for the dilution grade of urine. Urinary creatinine was determined by the method of Jaffè using alkaline picrate test with UV/Vis detection at 490 nm (30). Samples with creatinine concentrations lower than 0.3 g/L or higher than 3.0 g/L were excluded from statistical analysis according to the American Conference of Governmental Industrial Hygienists (ACGIH) recommendation (31).

Direct/Oxidative DNA Damage - Fpg Comet Assay

Specialized medical personnel collected, at end-shift of the third working day, whole venous blood samples from exposed workers by venipuncture in sterile heparinized disposable syringes and transferred them to the laboratory. We used (Fpg) modified Comet assay to measure direct and oxidative DNA damage. In particular, the Fpg is a glycosylase that recognizes and specifically cuts the oxidized bases (principally 8-oxoguanine) from DNA, producing apurinic sites converted in breaks by the associated AP-endonuclease activity detected by comet assay as Fpg sites estimating oxidative DNA damage. Lymphocytes were isolated on a Ficoll-based density gradient and suspended in 1 ml of PBS (without Ca^{2+} and Mg^{2+}), then the procedure of Collins et al. (32), with minor modifications (33) was used. For each subject two GelBond films (1 to be treated with Fpg enzyme and the other without) with a first layer of Normal-Melting Agarose (NMA) 1% in PBS and a second layer of cell sample (a few thousand) in low-melting agarose (LMA) 0.7% in PBS, were prepared. It allows the detection of direct DNA lesions (single – double strand breaks and alkali-labile sites) and oxidative DNA damage, respectively. The slides with gel bond were bathed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100, and 10% DMSO added fresh) and kept in the dark for 1 h at 4°C. Then they were washed 3 times in enzyme buffer (50 mM Na_3PO_4, 10 mM EDTA, 100 mM NaCl, pH 7.5), drained and incubated with 50 ml of either buffer or Fpg (1 mg/ml in enzyme buffer) in the dark for 30 min at 37°C. The slides were placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 40 min at 4°C to allow denaturing and unwinding of the DNA and the expression of alkaline-labile sites. Electrophoresis was done in the same buffer at 25 V and 300 mA for 30 min to allow the fragments of damaged DNA to migrate toward the anode. The slides were then washed 3 times with Tris/HCl 0.4 M for 5 min and stained with 50 ml ethidium bromide (10 mg/ml). Slides were examined by eye at 200X magnification under a fluorescence microscope. Images of 100 randomly selected comets for each slide were acquired and analyzed with a specific image analyzer software (Delta Sistemi, Roma, Italy). For each subject we calculated the mean values of tail DNA%, tail length (TL), and tail moment (TM). Tail DNA%

(ratio of intensity of the tail and total intensity of the comet) measures the number of broken pieces of DNA; TL (comet tail length) measures the smallest detectable size of migrating DNA (small DNA fragments with high capacity to migrate); TM (product of the tail length and the percentage DNA in tail) furnishes a measure of both the above parameters. We considered for each subject the mean value of Tail DNA%, TM and TL from enzyme-untreated cells to evaluate direct DNA damage.

Tail DNA%enz, TMenz, and TLenz from Fpg-enzyme treated cells evaluate total (direct and oxidative) DNA damage. We deducted tail DNA%, TM and TL from the tail DNAenz%, TMenz, and TLenz both in exposed and unexposed subjects to obtain oxidative DNA damage (Fpg sites).

The difference (tail DNAenz% - tail DNA%) was used to identify subject positive to oxidative DNA damage. In particular, subjects with mean values of the difference (tail DNAenz% - tail DNA%) exceeding a fixed arbitrary cut off value of 4 were considered positive to oxidative DNA damage.

Genotyping

Genomic DNA was isolated from the whole blood of the workers by using the QiAmp DNA blood mini kit cat. N. 51306 (Qiagen, Germany) following the manufacturer's instructions. Polymerase Chain Reaction (PCR) of *hOGG1 Ser*^{326}*Cys*, and *XRCC1 Arg*^{399}*Gln* was performed in the thermocycler (Multigene optimax thermal cycler, Aurogene SRL, Italy). Each reaction mixture contained 1X PCR buffer, 100 ng of DNA, 1 Unit of Taq polymerase per DNA sample (Promega), 0.3 μM of Forward and Reverse primers, 0.2 mM of dNTP and 2 mM of $MgCl_2$ in a total volume of 40 μl. Forward and reverse primers were purchased from Metabion GmbH (Germany-Dasit Carlo Erba-Italy). hOGG1 (F:ACTGTCACTAGTCTCACCAG, R:GGAAGGTGCTTGGGGAAT). Amplification conditions were: 95°C 7 min, 94°C 30," 60°C 30," 72°C 30," 72°C 7 min. XRCC1 (F:TTGTGCTTTCTCTGTGTCCA, R:TCCTCCAGCCTTTTCTGATA). Amplification conditions were 95°C 7 min, 95°C 30," 56°C 30," 72°C 1 min, 72°C 7 min. PCR products were separated on 1–2% agarose gel with TBE buffer (Tris, Boric acid, EDTA) (Cat. BMR 918100 Euroclone MI, Italy), and stained with gel red solution (Biotium CA, US). For each polymorphic gene, 20 μL of each amplicon were digested in 1X digestion buffer (New England Biolabs, MA, US and Thermo Fisher Scientifics) undergoing enzyme inactivation according to the procedure suggested by the manufacturer's instructions. 15U of Fnu4HI enzyme were used for *hOGG1* digestion 2 h at 37°C with the following restriction pattern: wt (Ser/Ser) 200 bp; het (Ser/Cys) 200, 107, 100 bp; mut (Cys/Cys) 107–100 bp. 15 U of MspI were used for *XRCC1* digestion overnight at 37°C with the following restriction pattern: wt (Arg/Arg) 374 and 221 bp; het (Arg/Gln) 615, 374, 221 bp; mut (Gln/Gln) 615 bp. Twenty μL of digested products of each polymorphic gene were run on agarose gel, stained with gel red solution to verify each fragment length.

Statistical Analysis

Analyses were carried out with SPSS/PC statistical software package 19.0 (Inc., Chicago, IL, USA) and statistical software R (R Foundation for Statistical Computing, Vienna, Austria).

TABLE 1 | Characteristic of the subjects with polymorphisms of the analyzed genes.

Subject	Task	Smoke	*hOGG1*	*XRCC1*
N1	Roller	NO	wt	wt
N2	Roller	YES	wt	wt
N3	Roller	NO	het	het
N5	Roller	NO	mut	het
N7	Roller	NO	het	mut
N10	Roller	NO	het	het
N11	Roller	NO	wt	mut
N12	Roller	YES	wt	wt
N13	Roller	NO	het	het
N15	Roller	na	wt	wt
N16	Roller	NO	wt	wt
N4	Spray	YES	wt	wt
N6	Spray	NO	wt	wt
N8	Spray	NO	mut	wt
N9	Spray	YES	wt	wt
N17	Spray	YES	mut	het
N18	Spray	NO	wt	het

Wt, wild type; het, heterozygous; mut, homozygous mutant.

The VOCs and their most important metabolites concentrations were measured as continuous variables. Normality of the distributions was assessed in according to the Kolmogorov–Smirnov tests. The significance level for all tests was $p < 0.05$ (two-tailed). Multivariate linear regression models were used to determine the statistical significance of the dose- response relationships in which the biomarkers of DNA damage, direct and oxidative, play the role of outcome variables and the dose biomarkers, both the unmetabolized VOCs and their metabolites, play the role of explanatory variables. The smoking habit and the age were introduced as confounders whilst the individual susceptibility, evaluated by means of the polymorphisms of the genes involved in detoxification, was considered among the explanatory variables. The gene polymorphism was treated as a three- or two-level factor. The fitted models were of the form lm (ox_stress_biomarker ∼ dose_biomarker + polymorphism) (1).

RESULTS

The workers were all male, 15 of Bengali ethnicity, one from Tunisia and another one from Iraq. Their age ranged from 21 to 49 years, with a mean of 39 years. The characteristics of the workers' sample are reported in **Table 1**.

Personal Air Monitoring

The external dose in terms of personal VOCs vapor concentration averaged over the 17 exposed subjects resulted: acetone 1.32 mg/m^3, ethyl acetate 7.28 mg/m^3, benzene 0.014 mg/m^3, toluene 34.1 mg/m^3, n-buthyl acetate 1.19 mg/m^3, ethylbenzene 12.9 mg/m^3, p-xylene 13.3 mg/m^3, m-xylene 33.0 mg/m^3, and o-xylene 11.22 mg/m^3. Each average value resulted well-below the corresponding Occupational Exposure Limit

TABLE 2 | VOCs' metabolites concentrations in the end – shift urine.

	MA (mg/g cr)	PGA (mg/g cr)	SPMA (μg/g cr)	2_MHIPP (mg/g cr)	3 and 4_MHIPP* (mg/g cr)	SBMA (μg/g cr)
Mean	7.33	4.40	1.73	12.33	57.31	14.70
Median	7.68	4.56	1.82	13.00	60.04	15.24
SD	8.09	4.75	1.66	13.73	63.21	15.40
5th perc	8.49	4.94	1.75	14.46	66.19	16.00
25th perc	8.50	4.81	1.87	14.69	65.85	15.90
75th perc	8.62	4.24	1.96	14.93	65.16	16.12
95th perc	8.94	4.42	2.08	15.67	67.13	16.67
Max	9.38	4.63	2.17	16.28	69.78	16.62
Min	7.93	4.30	1.81	13.44	65.49	16.55

**The metabolites 3 and 4 methtylhyppuric acids are not separated by the chromatographic process. However, the occupational exposure limit value refer to the sum of the three metabolites.*

TABLE 3 | Urinary unchanged VOCs concentrations in the end – shift urine.

	Ethyl acetate (μg/g cr)	Benzene (μg/g cr)	Toluene (μg/g cr)	n-butyl acetate (μg/g cr)	Ethylbenzene (μg/g cr)	p-xylene (μg/g cr)	m-xylene (μ g/g cr)	o-xylene(μg/g cr)
Mean	463.9	20.9	62.0	6.1	32.2	27.6	101.9	56.4
Median	58.8	6.5	11.9	4.0	10.1	6.9	34.1	25.5
SD	1252.7	39.1	169.9	6.5	63.1	59.1	201.3	83.3
5th perc	27.8	2.5	6.0	2.4	3.3	1.0	13.0	13.1
25th perc	41.8	3.5	8.4	3.1	7.0	4.0	22.2	18.9
75th perc	103.3	17.6	16.4	6.5	15.8	8.3	48.7	39.4
95th perc	2505.0	73.4	271.9	15.6	160.8	168.1	496.4	223.6
Max	5015.3	163.2	688.5	29.5	242.6	205.8	788.0	333.2
Min	23.8	2.4	5.9	2.1	2.6	0.7	12.8	12.9

Value in Italy (OELV), whereas the potential exposure to the mixture was found on average close to half of the mixture limit value. This last is evaluated by normalizing the exposure level to each VOC to its TLV and summing up the contribution of all the VOCs in the mixture. This sum must not exceed unity.

Biological Monitoring (Dose Biomarkers)

The urinary concentrations of the most specific VOCs metabolites are reported in **Table 2**. These values are all well-below the BEI (Biological Exposure Indexes) given by the ACGIH (400 mg/g Cr for the sum of PGA and MA, 25 μg/g Cr for the SPMA, 1.5 g/g Cr for the sum of the three MHIPPs and 300 μg/g Cr for the SBMA). In fact, although the exposure levels are quite close to the TLV for the mixture as regards the airborne concentration in the case of the spray painters, the personal protective equipment are able to reduce effectively the exposure. The VOCs' metabolites in **Table 2** are relative to the end-shift sampling. The concentrations at the beginning and the end of the work shift are significantly different at the level $p < 0.05$.

The mean urinary concentrations of the target unchanged VOCs measured at end of the work-shift, normalized to the creatinine concentration (μg/g cr), are reported in **Table 3**. On average, the maximum percentage increment between the beginning and the end of the work-shift occurred for the p-xylene, 22%, followed by the toluene, 14%.

Direct/Oxidative DNA Damage-Urinary Oxidized Bases (Effect Biomarkers)

In **Table 4** the distribution of the direct DNA damage biomarkers (Tail DNA%, TM and TL) and of the oxidative stress biomarkers are reported. These last consist of the systemic oxidative DNA damage biomarkers (Fpg sites) and the urinary DNA and RNA oxidation products, 8-oxoGuo, 8-oxodGuo and 8-oxoGua. The biomarkers of total damage to the DNA are also listed in terms of TL, TM and Tail DNA% in presence of enzyme treatment. Fpg-comet assay also showed that 10 out of 17 studied workers (58.8%) were positive to oxidative DNA damage. Tail DNA% and comet oxidative DNA damage of this study are higher than those found for unexposed subjects in other studies by the same authors, in which the genotoxic and oxidative effects in exposed workers were compared to control groups (17). As regards the urinary oxidized nucleic acid bases, they provide useful information mainly because they are very sensitive to gradients in the exposure levels. Indeed, a significant difference was measured between the beginning and the end of the work-shift. Data from

TABLE 4 | Biomarkers of direct and oxidative damage to the DNA.

	Direct DNA damage without Fpg enzyme			Total DNA damage direct and oxidative with Fpg enzyme			Oxidative DNA damage Fpg sites (enz-buff)			Urinary oxidized nucleic acid bases		
	Tail DNA %	TM (AU)	TL (μm)	Tail DNA % enz	TM enz	TL enz	Tail DNA %	TM	TL	8oxoGua (μg/g cr)	8-oxoGuo (μg/g cr)	8-oxodGuo (μg/g cr)
Mean	17.68	5.67	26.85	22.39	7.87	37.40	4.71	2.20	10.55	11.21	16.13	5.53
Median	16.30	5.68	27.50	22.70	7.93	39.00	4.30	2.03	10.20	6.20	14.72	5.59
SD	4.35	1.76	7.05	4.39	1.48	7.12	3.07	0.95	5.16	13.69	6.12	1.90
5th perc	12.42	3.34	18.51	15.68	5.51	25.68	0.48	1.08	4.93	0.01	10.04	3.28
25th perc	15.30	4.22	20.71	18.90	7.60	33.30	2.10	1.53	5.70	0.83	11.20	4.05
75th perc	20.90	7.23	29.80	24.43	8.60	40.60	6.50	2.66	12.90	14.84	18.33	5.98
95th perc	24.42	8.09	35.76	28.60	9.83	44.96	9.6	3.70	19.55	32.53	28.30	8.17
Max	26.50	8.32	44.00	31.40	10.35	54.00	11.60	4.09	20.30	46.63	31.16	10.71
Min	10.50	2.94	14.80	15.60	5.17	24.40	0.40	0.58	3.69	0.01	9.94	2.99

AU, arbitrary units.

the same group of exposed workers are reported in Tranfo (15) in which these differences can be appreciated. In particular, the 8-oxodGuo quite doubles due to daily VOCs' exposure.

The effect of the polymorphisms on the oxidative stress biomarkers of the Comet test was studied by means of a one-way ANOVA. Two polymorphisms are differently expressed in the genotypes of our sample, i.e., *hOGG1* and *XRCC1*. The polymorphism of the *XRCC1* is significantly associated to the Tail DNA% enz-buf. The distribution of this variable as function of the three variants, wild-type, heterozygous and mutant is shown in **Figure 1**.

Tail DNA % enz-buf is lower in the wild type than in the heterozygous and mutant variants, both resulting disadvantageous genotypes. The difference between the heterozygous and the wild type was not statistically significant.

VOCs Exposure-Oxidative Effect Relationship

As the dose biomarkers could be highly correlated among them due to the paint chemical composition, it is useful to analyze their correlation matrix, shown in **Table 5**.

We notice that the correlation between the dose biomarkers belonging to different groups, i.e., VOCs metabolites, airborne and urinary VOCs concentrations is in general within 0.80 except in the case of the airborne n-butyl-acetate and the urinary ethylbenzene and xylenes concentrations, for which $r^2 = 0.88$ and 0.86, respectively. The maximum correlation between the different metabolites is $r^2 = 0.80$, between MHIPP and MA. The airborne toluene is strongly correlated to the airborne ethylbenzene, $r^2 = 0.82$ and to the airborne xylenes concentration, $r^2 = 0.80$. The urinary toluene concentration is strongly correlated to the urinary concentration of ethylbenzene and xylenes, $r^2 = 0.80$ and 0.81, respectively. The airborne and urinary concentrations of ethylbenzene are both highly correlated, $r^2 = 0.99$, to the respective concentrations of xylenes.

The significance of the association between the VOC's urinary concentrations and the VOC's metabolites and the oxidative biomarkers is shown in **Table 6** in which the association between effect biomarkers and VOCs airborne concentration is also reported.

The model given by equation (1) was fitted with and without the effect of the *XRCC1* or *hOGG1* polymorphism. The oxidative stress biomarkers are oxidative DNA damage in terms of TL, TM and Tail DNA% (Fpg sites enzyme-buffer differences) as it was explained previously and DNA turnover urinary biomarkers.

A significant association was found between the oxidative DNA damage (TLenz-buf) and the 8-oxoGuo and the MHIPPs concentration. The association between MHIPPs and TLenz-buf is strengthened if the polymorphism of the gene *hOGG1* is added. TLenz-buf is significantly associated to the airborne xylenes, toluene, ethylbenzene and ethyl-acetate concentrations and these associations are all strengthened if the polymorphisms of *hOGG1* and *XRCC1* are added.

The 8-oxodGuo is significantly ($p = 0.039$) associated to the xylenes concentration in urine and to the toluene concentration in urine ($p = 0.0018$). When the factor representing the polymorphism is significant, it indicates that the wild type is significantly associated to lower levels of oxidative stress biomarkers than the heterozygous and mutant variants that are not significantly distinguishable.

VOCs Exposure-Direct DNA Damage Relationship

The direct damage to the DNA was evaluated by means of the genotoxicity biomarkers coming from the Comet test, i.e., TM, TL, and Tail DNA%. TM and the Tail DNA% were both significantly associated to the SBMA, urinary toluene metabolite. TL is associated to the toluene concentration in urine. The results of fitting the model (1) without the polymorphisms are shown in **Table 7**.

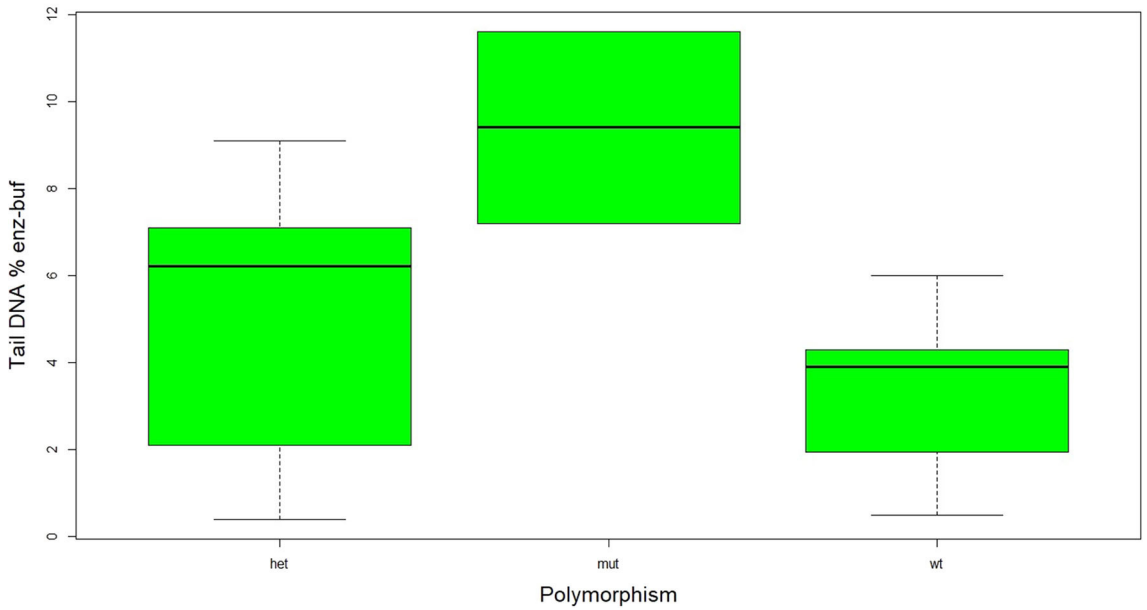

FIGURE 1 | Statistical distribution of the DNA percentage difference, representing the oxidative damage to the DNA in the three different genotypes of the *XRCC1* gene. The wild type is significantly lower than the mutant genotype. The variant of the *XRCC1* with respect to the wild type are disadvantageous types, both the heterozygous and the mutant.

TABLE 5 | Correlation matrix of the dose biomarkers: VOCs metabolites measured in urine, airborne and urinary VOCs concentrations.

	MA	PGA	SPMA	MHIPP	SBMA	Airborne ethylacetate	Airborne toluene	Airborne n_butil-acetate	Airborne ethyl benzene	Airborne xylenes	Urinary benzene	Urinary toluene	Urinary ethyl benzene	Urinary xylenes
MA (μg/g cr)	1.00	0.63	0.73	*0.80*	0.15	0.37	0.31	0.03	0.48	0.53	0.69	0.35	0.51	0.53
PGA (μg/g cr)		1.00	0.32	0.66	0.07	0.20	0.16	−0.06	0.18	0.24	0.32	0.46	0.28	0.28
SPMA (μg/g cr)			1.00	0.72	0.15	0.53	0.59	−0.03	0.54	0.54	0.54	0.30	0.28	0.31
MHIPP (μg/g cr)				1.00	0.20	0.61	0.58	−0.09	0.71	0.73	0.51	0.55	0.54	0.56
SBMA (μg/g cr)					1.00	0.59	0.52	−0.29	0.42	0.38	0.23	−0.25	−0.18	−0.12
Ethylacetate (mg/m^3)						1.00	*0.93*	−0.07	*0.88*	*0.87*	0.28	0.40	0.23	0.30
Toluene (mg/m^3)							1.00	−0.17	*0.82*	*0.80*	0.02	0.45	0.09	0.15
n_butyl-acetate (mg/m^3)								1.00	0.20	0.24	−0.04	0.69	*0.88*	*0.86*
Ethylbenzene (mg/m^3)									1.00	*0.99*	0.27	0.75	0.59	0.62
Xylenes (mg/m^3)										1.00	0.30	*0.80*	0.65	0.69
Benzene (μg/g cr)											1.00	−0.11	0.74	0.73
Toluene (μg/g cr)												1.00	*0.80*	*0.81*
Ethylbenzene (μg/g cr)													1.00	*0.99*
Xylenes_ (μg/g cr)														1.00

The biomarkers of direct damage to the DNA were found not significantly associated to the polymorphism of the *hOGG1* and *XRCC1*. In **Table 7** the β coefficient of the regressions, the increment of the effect biomarkers when the dose biomarker is incremented by a unit, and its standard error are also shown.

The linear regression of the DNA percentage in the tail and the SBMA is shown in **Figure 2**.

The regression is statistically significant and its R2 is 0.30. The VOCs' metabolites of this study are much higher than those found in smokers as regards the MA, PGA, MHIPPs (34). Differently SPMA and SBMA are about one half the average concentrations found by Lorkiewicz in a population of smokers. However, the effect of the smoking habit in the associations between the direct DNA damage and the SBMA was not significant. This result is confirmed by the fact that the cotinine is not significantly associated to the biomarkers of direct damage to the DNA.

TABLE 6 | Statistical association between the dose biomarkers and the biomarkers of oxidative stress.

Oxidative stress biomarker	Dose biomarker	Model (1) without polymorphism	Model (1) with hOGG1 polymorphism	Model (1) with XRCC1 polymorphism
TLenz – buffer (μm)	MHIPPs (μg/g cr)	$p = 0.034$	MHIPPs $p = 0.028$ *hOGG1* $p > 0.05$	MHIPPs $p = 0.04$ *XRCC1* $p > 0.05$
8-oxoGuo (μg/g cr)		$p = 0.041$	Ns	MHIPPs $p = 0.04$ *XRCC1* $p > 0.05$
TLenz – buffer (μm)	Airborne xylenes (mg/m^3)	$p = 0.049$	Air_xylenes $p = 0.04$ *hOGG1* $p > 0.05$	Air_xylenes $p = 0.019$ *XRCC1* $p = 0.026$
TLenz – buffer (μm)	Airborne toluene (mg/m^3)	$p = 0.006$	Air_toluene $p = 0.0017$ *hOGG1* $p = 0.034$	Air_toluene $p = 0.0023$ *XRCC1* $p = 0.056$
TLenz – buffer (μm)	Airborne ethylbenzene (mg/m^3)	$p = 0.049$	Air ethylbenzene $p = 0.032$ *hOGG1* $p > 0.05$	Air_ethylbenzene $p = 0.015$ *XRCC1* $p = 0.02$
TLenz – buffer (μm)	Airborne ethylacetate (mg/m^3)	$p = 0.023$	Air ethylacetate $p = 0.012$ *hOGG1* $p > 0.05$	Air ethylacetate $p = 0.012$ *XRCC1* $p > 0.05$
8-oxodGuo (μg/g cr)	Urine p-xylene (μg/g cr)	$p = 0.039$	Ns	ns
8-oxodGuo (μg/g cr)	Urine toluene (μg/g cr)	$p = 0.0018$	Ns	ns

The model (1) was fitted with or without the polymorphisms.

TABLE 7 | Statistical association between dose biomarkers and biomarkers of direct DNA damage.

Direct DNA damage biomarker	Dose biomarker	*p*-value	β coefficient	Standard error
TM	SBMA (μg/g cr)	0.045	0.13	0.06
Tail DNA %		0.023	0.36	0.14
TL (μm)	Urine toluene (μg/g cr)	0.037	−0.022	0.009

VOC Exposure-Total (Direct and Oxidative) DNA Damage Relationship

In **Table 8** the significance of the associations between the biomarkers of total damage to the DNA, i.e., the tail length, tail moment and DNA percentage in presence of the Fpg digestion enzyme, and the dose biomarkers are listed.

Tail DNA % enz is significantly associated to the SBMA, this regression seems to be dominated by the direct effect. In the case of the TLenz the association with MHIPPs becomes significant when the polymorphism of the *XRCC1* is added to the model. The TMenz is significantly associate to the SBMA if the *XRCC1* polymorphism is kept into account into the model.

A statistically significant association was found between the total DNA damage biomarker TMenz and the polymorphism of the gene *XRCC1* with the wild type significantly ($p = 0.026$) lower than the heterozygous (**Figure 3**).

The association between the comet assay total DNA damage biomarkers and the urinary nucleic acid oxidation biomarkers were studied. A significant ($p = 0.042$) association was found between the 8-oxoGuo concentration, representing the RNA oxidation products at the end of the metabolic path, coming from the RNA turnover, and the Comet test tail length in presence of the digestion enzyme. This last permits the evaluation of the oxidation processes. In fact, the digestion enzymes transform the damage to breaks so amplifying the DNA quantity in the tail of the comet test. The oxidation products of the purines and pyrimidines are transformed in DNA breaks by the digestion enzymes, endonuclease III and Fpg, respectively (35). The association between the biomarker of total, direct and oxidative damage to the DNA, and the 8-oxoGuo is shown in **Figure 4**.

Similar significant associations were found between the TLenz and the 8-oxoGuo and the 8-oxoGua evaluated at the beginning of the work-shift.

DISCUSSION

In the occupational setting, such as in the naval ship painting, professional workers are daily exposed to different mixtures of VOCs which are extremely toxic and carcinogenic. Although the risk of inhalation and dermal contact is strictly controlled by the use of suitable PPEs, a minimal amount of these compounds may be absorbed and metabolized by the body enzymes, resulting in generalized toxicity and damage of nucleic acids. The DNA lesions may occur particularly on the guanine, the nitrogen base most sensitive to oxidation and mutagenicity (16).

The main objective of the present work was to find out quantitative relations between the dose biomarkers and the biomarkers of damage, direct or oxidative or both, to the DNA. In order to do that we designed dose–response curves, in which the effect biomarkers are considered as outcome variables and the dose biomarkers as explanatory ones. The dose–response relationships permit to quantitatively evaluate a very early risk curve with respect to genotoxic

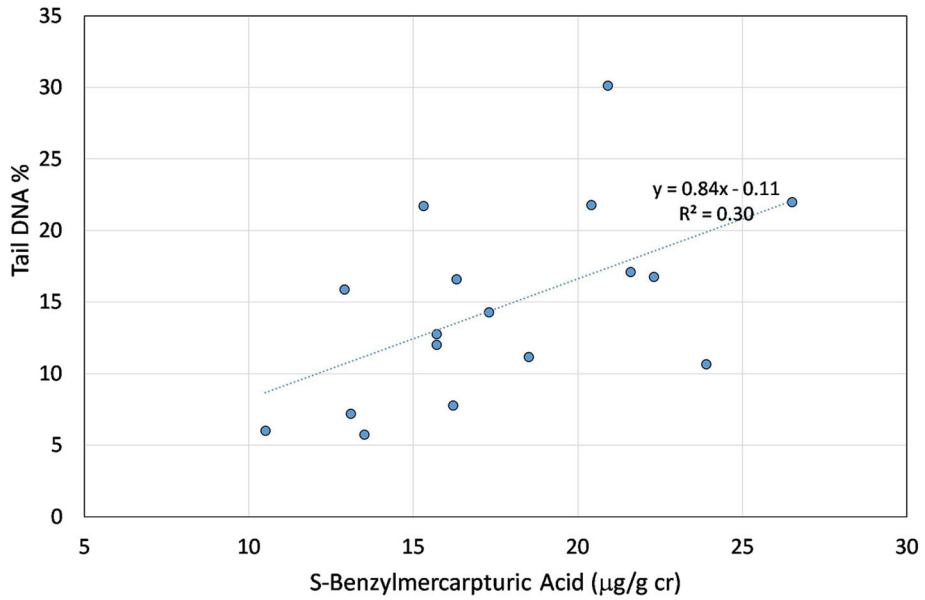

FIGURE 2 | Linear association between the S-Benzylmercapturic acid concentration and percentage of the DNA in the tail used as biomarker of direct damage to the DNA.

damage. As regards the oxidative stress both oxidative DNA damage and nucleic acid oxidation products at the end of the metabolic path, 8-oxoGuo and 8-oxodGuo, were kept into account.

Significant associations were found between oxidative DNA damage in terms of Tail length (the most sensible comet parameter to show the number of generated fragments in the case of low/moderate DNA oxidation), the 8-oxoGuo and the Methylippuric acids concentration ($p = 0.034$). This last are specific metabolite of xylenes, which represent one of the most important components of the mixture that workers are exposed to, the other one being the toluene. The 8-oxodGuo was also found significantly associated to the Methylippuric acids. The 8-oxodGuo, coming from the DNA repair and turnover, was also found significantly associated to the urinary concentrations of both xylenes and toluene. The association between MHIPPs and oxidative DNA damage (TLenz-buf) is strengthened if the polymorphism of the gene *hOGG1* is added. Higher level of urinary biomarkers of nucleic acid oxidation in particular 8-oxoGuo, correlating with internal exposure metabolites MA+PGA, have reported by Manini et al. (36) in styrene-exposed workers employed in two plastic lamination plants in comparison to controls. The authors concluded that styrene exposure seems to be associated with oxidation to nucleic acids, particularly to RNA and with induction of basic excision repair (BER) system (36). RNA is single-stranded and its bases aren't protected by hydrogen bonds or structural proteins and may be more susceptible to oxidative insults than DNA (37). Moreover, the presumable intra-cytoplasmatic ROS increase related to VOC induced oxidative stress is compatible with secondary oxidation of RNA molecules located in the cytoplasm and it could explain the 8-oxoGuo excretion associated with xylene exposure.

TABLE 8 | Statistical association between dose biomarkers and biomarkers of total (direct and oxidative) damage to the DNA.

Total DNA damage biomarker	Dose biomarker	Model (1) without polymorphism	Model (1) with XRCC1 polymorphism
TLenz (μm)	MHIPPs (μg/g cr)	ns	MHIPPs $p = 0.049$ *XRCC1* $p = 0.05$
TMenz	SBMA (μg/g cr)	ns	SBMA $p = 0.039$ *XRCC1* $p = 0.007$
Tail DNA% enz		$p = 0.025$	SBMA $p = 0.015$ *XRCC1* $p > 0.05$

Only the significant relations are reported. The model (1) was fitted with and without the polymorphism of the gene XRCC1.

As the xylenes, toluene, ethylbenzene and ethylacetate airborne concentrations are strongly correlated, $r^2 > = 0.80$, the causal implications of the statistical associations with TLenz-buf reported in **Table 6** cannot be univocally assessed. In other words, only one or two of the above-mentioned chemicals could be causally related to the DNA oxidative damage effect, while the others could just be correlated due to the paint composition. On the other hand, the observation that only the xylenes urinary metabolite (MHIPP) is correlated with TLenz-buf suggests that the causal association likely involves the xylenes, while for the other chemicals no conclusion can be drawn. Similarly, the correlations with the 8-oxodGuo of the strongly correlated toluene and xylenes urinary concentrations, cannot be attributed to one or the other chemical, but the relation of 8-oxodGuo with DNA repair mechanisms suggests that the

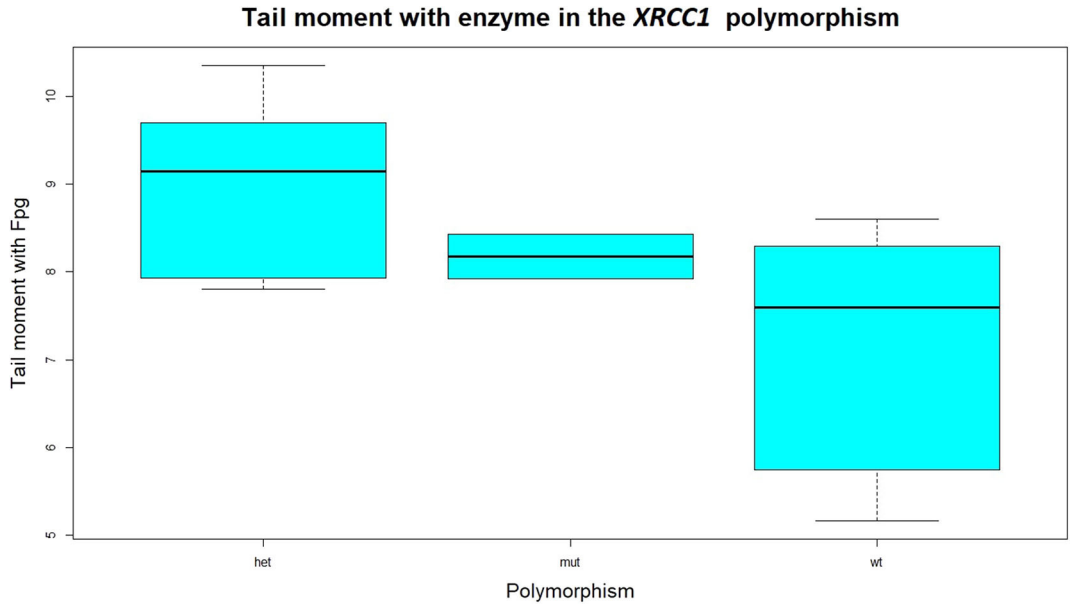

FIGURE 3 | Statistical distribution of the tail moment with enzyme, representing the total DNA damage, direct and oxidative in the three different genotypes of the *XRCC1* gene. The wild type is significantly lower than the heterozygous and the mutant genotypes. The difference between mutant and heterozygous is not statistically significant. The variant of the *XRCC1* with respect to the wild type are disadvantageous types, both the heterozygous and the mutant.

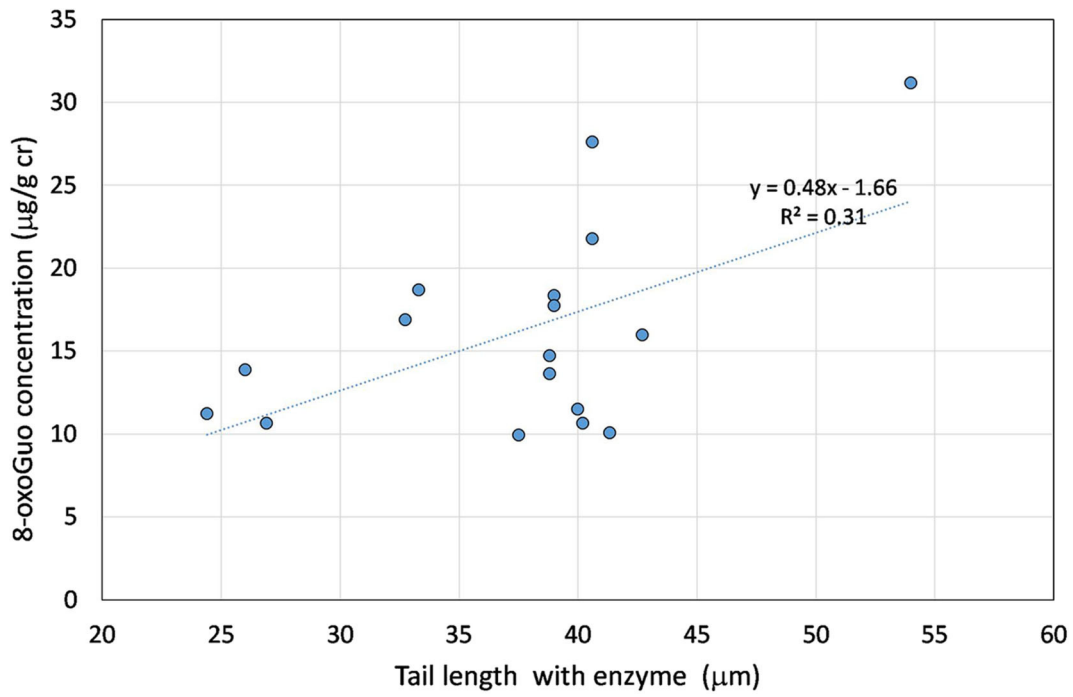

FIGURE 4 | Linear association between the tail length in presence of the formamidopyrimidine glycosylase enzyme and the 8-oxoGuo concentration. The statistical association between the two variables is statistically significant ($p = 0.042$).

main causal role is probably attributable to the toluene, whose metabolite SBMA was found associated to a direct damage to the DNA (**Table 7**).

Definitely our findings confirm the induction of oxidative nucleic acid damage in association with xylene and toluene exposure found either in blood and urine.

All data achieved during the biomonitoring campaign take into account the contribution of specific polymorphic enzymes, which are involved in the metabolism of the chemical compounds with different efficacy and depending on their genetic background. In particular, we took into account the *hOGG1 Ser326Cys* and *XRCC1 Arg399Gln* polymorphic genes, since they are relevant for the maintenance of the DNA stability (38, 39). The polymorphism as unique factor was significant only in the case of the *XRCC1* gene. Statistical significant associations were found between the *XRCC1* polymorphism and oxidative DNA damage in terms of Tail DNA % enz-buf. The *XRCC1* was also significant in explaining the TMenz related to the total, direct and oxidative damage to the DNA. The presence of the polymorphism as second factor affecting the dose-response relationships often strengthens the significance of the association between the dose and the effect biomarkers. In general, the wild type genotype of both the *XRCC1* and *hOGG1* was found significantly associated to lower levels of damage to the DNA biomarkers, the heterozygous and mutant genotypes being not significantly distinguishable. The conclusion is that the variants, with respect to the wild-type, of both *XRCC1* and *hOGG1* are disadvantageous, meaning that subjects with wild-type genotype (53% of the workers in the case of *XRCC1*) have more functional DNA repair ability compared to the other two genotypes, whose capability in the maintenance of DNA stability is less efficient. The *XRCC1* and *hOGG1* polymorphisms can be considered as susceptibility indices that could be kept into account in the occupational risk evaluation.

In addition to the oxidative DNA damage, the direct one was kept in consideration. A significant association was found between Tail DNA% and TM biomarkers with SBMA, specific metabolite of the toluene, while the TL biomarker was significantly associated to the urinary toluene concentration. Direct DNA damage by comet assay in painters exposed to relatively low toluene levels was also found, together with lipid peroxidation, by Moro et al. (11). The same authors showed in a previous study increased levels of oxidative stress biomarkers Malondialdehyde (MDA), superoxide dismutase (SOD) and Catalase (CAT) in industrial painters exposed to toluene, xylene, styrene, ethylbenzene and lead suggesting toluene as the principal factor responsible for increased lipid peroxidation (10).

One interesting finding of our work is that the toluene is mainly associated to the biomarkers of direct damage in the Comet test whilst the xylenes are significantly associated to the biomarkers of oxidative stress. As regards the associations between the urinary oxidative stress biomarkers and those coming from the Comet test, a significant relation was also found between the TLenz, representing a total DNA damage, and the 8-oxoGuo, particularly related to the RNA turnover and repair. Some caution must be taken in interpreting the level of specificity of the dose response relations, as a multicollinearity problem occurs between the different VOCs, In addition, it is not possible to exclude an interaction, possibly synergistic, between the different solvents in inducing an oxidative or direct damage to the DNA. Although this limitation should always be considered, this research could represent a useful paradigm for further investigations, to find out useful associations between solvent exposure and different dose and effect biomarkers, contributing in the mitigation of the health risk in the occupational setting.

CONCLUSIONS

In this study, specific dose–response relationships are proposed, which permit the quantitative evaluation of very early risk curves with respect to genotoxic damage in workers exposed to a mixture of VOCs. Such curves relate dose biomarkers, i.e., unchanged VOCs and their metabolites, to direct or oxidative damage to the DNA. This study suffers from the main limitation that the different VOCs are all correlated among them. As consequence, the results should be interpreted with caution, because the correlation between the different VOCs does not permit a rigorous evaluation of each separate effect. The dose-response relations found in this study hold in a range of mild VOCs exposure, showing that, also in this range, some damage to the DNA, both direct and oxidative, does occur. Occupational exposure to chemical agents has been demonstrated to produce a measurable level of oxidatively generated damage to DNA and RNA, which is repairable only in the case of DNA. Effects of workplace exposure to asbestos, benzene, fine particulate matter, polycyclic aromatic hydrocarbons, silica, metals, styrene, toluene, and xylenes on the level of urinary 8-oxodGuo have been reported in the literature.

Even in conditions which are regarded as not dangerous, below the occupational exposure limit values, there is a detectable increase in the biomarkers concentration after a working shift, even if this is still within the range measured in the general population (15).

Early effect dose–response relations are crucial at the aim of preventing diseases resulting from a long-term low–dose exposure to VOCs. In more detail, linear regression models showed significant associations between oxidative stress biomarkers, i.e., oxidative DNA damage (TLenz-buffer difference), the 8-oxoGuo urinary concentration, and the Methylhippuric acids, specific metabolite of the xylenes. Oxidative DNA damage in terms of TL was found also significantly associated to the airborne xylenes, toluene, ethylbenzene and ethylacetate concentrations, whilst the 8-oxodGuo concentration was significantly associated to the urinary concentration of both xylenes and toluene. These results confirm that oxidative DNA damage by Fpg-comet and both urinary 8-oxodGuo and 8-oxoGuo represent valuable biomarkers for the biomonitoring of occupational exposure to VOCs, also at low concentration levels. In particular, these findings seem to suggest that 8-oxoGuo, related to RNA oxidation, and TL are the most sensitive biomarkers to evaluate early and still reparable oxidative effects of occupational exposure in painters, even at levels well-below the Biological Exposure Indexes.

In the case of the biomarkers of direct damage to the DNA, significant associations were found between Tail DNA%, TM and the SBMA toluene metabolite, and between TL and urinary concentration of toluene.

The study of the polymorphism of the XRCC1 gene on the direct and oxidative damage to the DNA showed that the oxidative DNA damage in terms of tail DNA% in the wild

type is significantly lower (meaning a lower level of oxidative stress) than in the other two genotypes, heterozygous and mutant. As regards the total (direct plus oxidative) damage to the DNA, TMenz was found significantly lower in the wild type than in the other two genotypes. The wild type of *XRCC1* seems to be associated to lower levels of damage to the DNA being the heterozygous and the mutant both disadvantaged and not significantly different between them. The *hOGG1* was found not significantly associated to the DNA damage biomarkers when it is considered as the only factor. When multivariate mixed effects regressions are considered in which the polymorphisms are added to the dose biomarkers, the effect of the *hOGG1* polymorphism becomes significant, in the association between oxidative DNA damage in terms of TL and the airborne toluene.

As regards the polymorphism of *XRCC1* when it is added to the dose biomarkers it becomes significant in the case of the total DNA damage biomarkers and in particular in the associations between TMenz, TLenz, and MHIPPs or SBMA. In general, the polymorphisms of both *XRCC1* and *hOGG1* are associated to a larger susceptibility to the total, direct and oxidative, damage to the DNA confirming their interaction in Basic Excision Repair (BER) system, the main repair mechanism of DNA damage including oxidative one.

These findings confirm the role of genetic polymorphisms of genes involved in oxidative stress and DNA repair as biomarkers of individual susceptibility to xenobiotics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ASL (Local Sanitary Agency) of Region Marche. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS: conceptualization and data analysis. BB and DCar: subject enrollment and questionnaire administration. GT, MG, DCar, DP, MG, and NL'E: data collecting. GT, DP, and EP: VOCs' metabolites chemical analysis and DNA and RNA oxidation products analysis. MG and AG: VOCs chemical analysis as airborne and urinary concentrations. DCav, CU, AF, AC, and RM: genotoxicity and oxidative DNA damage biomarkers. PCh and PCa: polymorphisms analysis. RS, PCh, PCa, and DCav: manuscript writing. All authors reviewed and approved the manuscript.

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Exposure to Mild Steel Welding and Changes in Serum Proteins With Putative Neurological Function—A Longitudinal Study

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Welders are exposed to high levels of metal particles, consisting mainly of iron and manganese (Mn) oxide. Metal particles, especially those containing Mn can be neurotoxic. In this exploratory study, we evaluated associations between welding and expression of 87 putative neurology-related proteins in serum in a longitudinal approach. The study cohort from southern Sweden included welders working with mild steel ($n = 56$) and controls ($n = 67$), all male and non-smoking, which were sampled at two timepoints (T1, T2) 6-year apart. Observed associations in the longitudinal analysis (linear mixed models) were further evaluated (linear regression models) in another cross-sectional sample which included welders ($n = 102$) and controls ($n = 89$) who were sampled only once (T1 or T2). The median respirable dust levels for welders after adjusting for respiratory protection was at T1 0.6 (5–95 percentile: 0.2–4.2) and at T2 0.5 (0.1–1.8) mg/m^3. The adjusted median respirable Mn concentration was at T2 0.049 mg/m^3 (0.003–0.314) with a Spearman correlation between adjusted respirable dust and respirable Mn of $r_S = 0.88$. We identified five neurology-related proteins that were differentially expressed in welders vs. controls in the longitudinal sample, of which one (nicotinamide/nicotinic acid mononucleotide adenylyltransferase 1; NMNAT1) was also differentially expressed in the cross-sectional sample. NMNAT1, an axon-protective protein linked to Alzheimers disease, was upregulated in welders compared with controls but no associations were discerned with degree of exposure (welders only: years welding, respirable dust, cumulative exposure). However, we identified five additional proteins that were associated with years welding (GCSF, EFNA4, CTSS, CLM6, VWC2; welders only) both in the longitudinal and in the cross-sectional samples. We also observed several neurology-related proteins that were associated with age and BMI. Our study indicates that low-to-moderate exposure to welding fumes is associated with changes in circulating levels of neurology-related proteins.

Keywords: neurotoxicity, particle, manganese, Parkinson, NMNAT1

INTRODUCTION

It is well-established that high exposure to manganese (Mn) is neurotoxic. Mn can accumulate in the brain and cause the condition manganism, a neurological syndrome with symptoms similar to Parkinson's disease (referred to as Parkinsonism) including tremor, body rigidness, reduced smell, and impaired motor function and balance (1). Exposure to Mn has also been associated with Alzheimer's disease in a study combining human, *in vitro* and animal experiments (2). Welders have for a long time been considered a risk group of high Mn exposure due to the release of respirable Mn particles during welding (3, 4). Several studies have indicated that exposure to Mn in occupational settings is associated with neurological and neuropsychological health effects, higher prevalence of Parkinsonism, and increased Mn in brain as observed by magnetic resonance imaging scans (5–7), whereas other studies that have found no association (8, 9).

In Sweden, around 13,000 workers are registered as full-time welders of steel (10). Measurements during 2003 to 2005 at 10 different Swedish workplaces revealed that many welders were exposed to Mn levels above the occupational exposure limit (OEL) at that time (0.1 mg/m^3) and that the average exposure of all welders (0.08 mg/m^3, $n = 108$) was near the OEL (11). It was also observed that welders were still performing a major part of the work without appropriate protection (12). The current OEL for Mn in Sweden is 0.05 mg/m^3 (8-h total weight average - TWA) as respirable fraction (13). This is in line with the OEL in the European Union (14) while other countries, such as Germany and Finland, have a lower OEL of Mn in the respirable fraction (0.02 mg/m^3) (13).

In addition to Mn ions, exposure to metal nanoparticles in the welding fumes may also cause neurotoxic effects. *In vitro* studies indicate that neurotoxicity (e.g., cell death and oxidative stress) in neuronal cell models can be induced by both Mn oxide nanoparticles (15) and iron oxide nanoparticles (16, 17). Evidence suggests that following inhalation, metal (nano)particles are able to directly translocate to the brain via the olfactory bulb and sensory nerve endings in the respiratory tract (18–20).

It is important to clarify if current exposure levels to welding fumes cause neurotoxicity as well as to identify biomarkers of neurological changes. In this study, we investigated if welders show alterations in serum levels of a panel of proteins putatively related to neurobiological processes and neurological diseases.

METHODS AND MATERIALS

Study Design

This study is based on a cohort of welders established during 2010-2011 (timepoint 1) in the south of Sweden (21). The inclusion criteria were that the participants should be males and non-smokers for the last 6 months. At baseline, we recruited 101 welders working in 10 small- and medium-size welding companies and 127 age-matched controls working in medium-size companies (mainly food business) and municipalities (working as janitors or gardeners), all without occupational exposure to welding fumes or particles from other sources. The follow-up after 6 years was performed during 2016–2017 (timepoint 2) and included welders from 9 of the initial 10 companies (one of the companies had closed). There was a drop-out of 23% ($n = 23$) among the welders and 24% ($n = 31$) among the controls at timepoint 2 and the reasons were: sick on the medical examination day, declined participation without giving any particular reason, or had died since timepoint 1. At timepoint 2, we also recruited new participants (67 welders and 38 controls) with the same inclusion criteria. Overall, the timepoint 2 survey included examination of 145 welders (78 welders re-examined) and 134 controls (96 controls re-examined). Among welders participating at both timepoints, í out of 78 had retired or quit working due to illness since timepoint 1, and another 12 were employed at the same company as before but were no longer actively involved in welding. Those seven that had quit working were invited to visit either their old company or the clinic for the follow-up examination.

In this study, the participants were divided into two groups: one group with repeated measurements 2010/2011 and 2016/2017, and one group with measurements performed at either 2010/2011 or 2016/2017, i.e., cross-sectional group (90 welders and 69 controls). The latter group was used to see if we could replicate the associations observed in the group with longitudinal data. In the cross-sectional group, 8 welders and 7 controls were excluded from the analysis due to missing/poor quality data for the protein measurements. In addition, 27 controls and 20 welders were re-attributed to the cross-sectional group due to missing/poor quality data for the protein measurements for one of the timepoints. Further, 2 welders as well as 2 controls were excluded due to missing/poor quality data for the protein measurements at both timepoints. This amounts to a total of 123 individuals in the cohort group (56 welders and 67 controls) and 191 individuals in the cross-sectional group (102 welders and 89 controls). A flow diagram of the study design is shown in **Figure 1**. A similar study design on the same cohort of Swedish welders was used to evaluate the associations between cancer-related proteins in serum (22).

The participants filled out a questionnaire regarding country of birth, education, medical history, personal/family history of cancer, diet, physical activity, current as well as previous smoking history, use of snus (Swedish moist tobacco), alcohol consumption, current residence, and exposure to particles/smoke (e.g., welding fumes, dust, engine exhaust, diesel engine) during leisure activities. The inclusion criteria were that the participants should be non-smokers for the last 6 months. However, based on the questionnaire data, we identified at follow-up a few individuals that actually were current smokers.

Blood samples were collected in the same way at both timepoints in BD vacutainers for serum, allowed to clot at room temperature for 10 min and then centrifuged at 2,400 rcf for 10 min. Upon separation, serum samples were aliquoted and kept on dry ice for transportation to the laboratory in the Division of Occupational and Environmental Medicine at Lund University, and then stored at −80°C until analysis. Samples were shipped on dry ice to Uppsala for protein analysis.

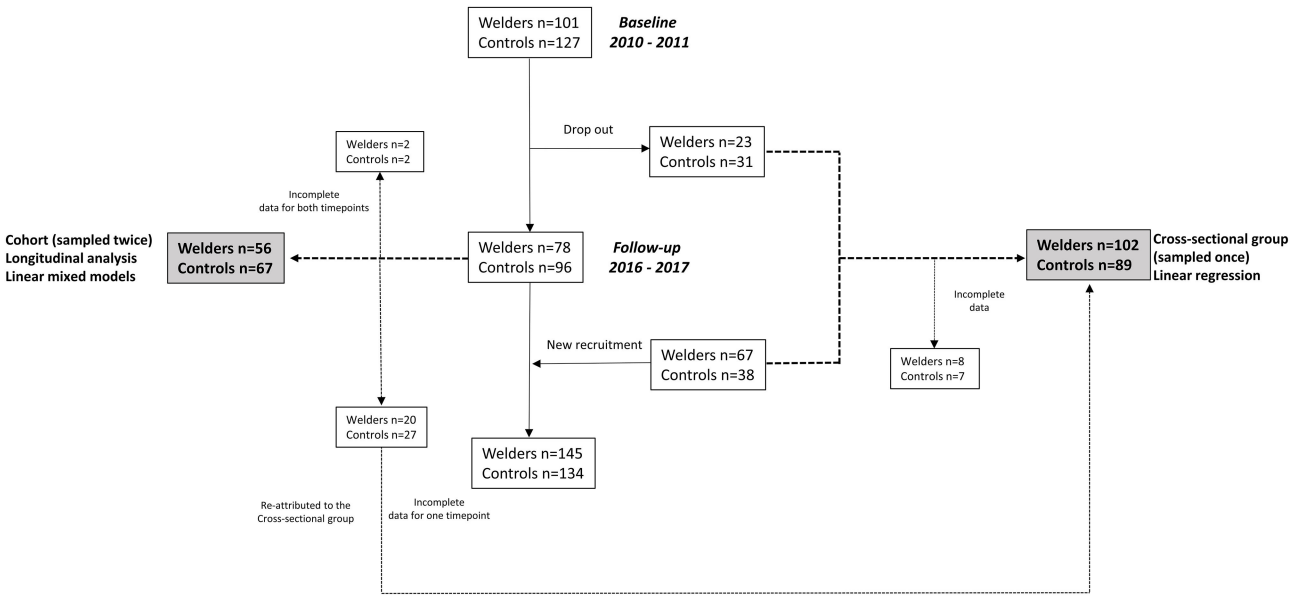

FIGURE 1 | Flow-chart of the study design. Incomplete data refers to low quality of the protein data.

Exposure Assessment

Both for welders and controls, we used a structured questionnaire inquiring about the present and past workplaces, type and duration of work, and explicitly whether they were exposed to welding or diesel fumes at past or present workplaces. For welders, we also asked questions about the type of welding, total hours of welding during the usual working week, place of welding, area level or point source exhaust use, as well as use of personal respiratory, noise, and eye protection devices while welding.

Personal Respirable Dust Measurement

Personal exposure measurement of respirable dust was performed for the active welders and stationary area-monitoring of respirable dust was conducted for the controls. For personal sampling, a cyclone (BGI4L, BGI, Mesa Labs, USA; cut-off = 4 μm) was used for collecting respirable dust. The cyclone was fitted with a filter cassette, containing 37-mm mixed cellulose ester filters with an 0.8-μm pore size (pre-weighed) and was placed within the breathing zone of each welder. The airflow through the sampler was set to 2.2 L/min and regularly checked before, during, and after sampling with a flow meter (TSI Model 4100 Series, TSI Incorporated, USA). Personal sampling was performed during one work day and was coordinated with each company's shift working hours: the average sampling duration was approximately 7 h for both timepoints. It was not feasible to perform measurements over multiple days due to the large number of participating welders. Measured dust concentrations were corrected if respiratory protection was used: the measured concentration (outside respiratory protection) was divided by 3 as a correction factor to reflect the actual exposure level (11, 21). At timepoint 2, one welder used a half-mask, for which a correction factor of 2 was used instead, and another four welders used newer versions of powered air-purifying respirators with double visors, for which a factor of 50 was used (personal communication with Karlsson J-E, occupational hygienist, Clinic of Occupational and Environmental Medicine, Lund University Hospital, Sweden). The filter samples were analyzed gravimetrically according to a validated method for determination of respirable dust (23). The limit of detection was set to 0.05 mg/sample.

For welders with incomplete exposure data, the individual respective exposure level was estimated using geometric mean exposure data obtained from welders working at the same workstation, engaged in similar tasks, or in the same company. The use of protection devices was then corrected for as described above. For two welders, to fill missing data for the exposure assessment, exposure data previously collected at the welding companies (11, 21) were also used. Only active welders (i.e., not retired or welders with non-welding work tasks) had either measured or assessed respirable dust data. In the end, 56 welders had respirable dust data at both timepoints (timepoint 1: measured $n = 28$, estimated $n = 28$; timepoint 2: measured $n = 46$, and estimated $n = 10$).

For the controls at timepoint 1, full-shift personal breathing zone samples of respirable dust were collected from two companies for 19 control subjects. From four companies, area-level air pollution monitoring of respirable dust was performed using a direct reading monitor, SidePak Model AM510 (TSI Incorporated) with a Dorr-Oliver cyclone (21). At timepoint 2, stationary area monitoring of respirable dust fractions was performed using a DustTrak DRX monitor (TSI Incorporated). At both timepoints, these monitors were placed at breathing zone height in the area where workers spent the most time during their work shifts. On average, the monitoring of each control lasted approximately 4 h at each company's work site.

In companies where workers spent time at two different workstations, measurements were taken in both areas, and a time-weighted average of the two sites was calculated.

Cumulative Dose

For timepoint 1, the cumulative dose was estimated by multiplying the value for respirable dust at timepoint 1 (adjusted for respiratory protection) with number of welding years at timepoint 1. Cumulative dose for timepoint 2 was calculated by multiplying respirable dust (adjusted for respiratory protection) at timepoint 2 with welding years at time point 2 and adding the resulting product to the cumulative dose for timepoint 1.

$$\begin{aligned}&\textit{Cumulative dose timepoint\_1}\\&\quad = \textit{Respirable dust timepoint\_1} \times \textit{Years welding timepoint\_1}\\&\textit{Cumulative dose timepoint\_2}\\&\quad = \textit{Cumulative dose timepoint\_1} + [\textit{Respirable dust timepoint\_2}\\&\quad \times (\textit{Years welding timepoint\_2} - \textit{Years welding timepoint\_1})]\end{aligned}$$

Analysis of Metals on Respirable Dust Filters

Filters from timepoint 2 ($n = 104$ in total, out of which $n = 100$ are included in this study) were analyzed for element concentrations including Mn, and iron (24). After weighing, the filters were digested in 1 mL of concentrated nitric acid at 70°C for 16 h. After dilution with Milli-Q water, the metal concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS; iCAP Q, Thermo Scientific GmbH, Germany) in collision cell mode, with kinetic energy discrimination, using helium as the collision gas. Detailed information on the metal content and the quality control of the analysis is included in **Supplementary Table 1**.

Protein Measurement

Using the Proximity Extension Assay (PEA) technology (Olink Proteomics, Uppsala, Sweden) the serum samples were analyzed for 92 unique proteins related to neurobiological processes and neurological diseases (Neurology panel). The selected proteins have the following gene ontology terms: axon development ($n = 17$), axon guidance ($n = 12$), cell adhesion ($n = 36$). cell death ($n = 21$), cell differentiation ($n = 37$), cell growth ($n = 13$), cell metabolic process ($n = 42$), immune response ($n = 22$), MAPK cascade ($n = 15$), neurogenesis ($n = 26$), proteolysis ($n = 8$), signal transduction ($n = 48$), synapse assembly ($n = 6$), and other gene ontology terms ($n = 11$). One μL serum sample was used for the analysis. Processing, quality control as well as normalization were performed as previously described (25). Protein levels were reported as normalized protein expression (NPX) values on a log2-scale. The cut-offs for intra- or inter-assay CVs were <20% (calculated on linear data). In addition, proteins with 10% or more of the samples ≤ limit of detection were excluded from the analysis. Overall, out of the 92 proteins, 5 proteins (BDNF, GDNF, HAGH, BMP4, PLXNB1) were excluded due to low quality of the protein analysis; the statistical analyses were performed on 87 proteins only. The quality control of individual samples (pass/fail) was based on specific incubation and detection controls.

Statistics

All statistical analyses were completed by using R statistical software version 3.4.4 (R Foundation for Statistical Computing. Vienna. Austria).

Evaluation of Differences Between the Study Groups

Characteristics are presented as median and 5–95 percentile for the continuous variables and percentage for categorical variables. Differences between groups were evaluated with the Kruskal–Wallis rank sum test (followed by Dunn's *post-hoc* test) (when comparing three groups), paired samples Wilcoxon test (when comparing two groups) for continuous variables, and Fisher's exact test for categorical variables.

Data Exploration Using Principal Component Analysis

PCA heatmaps were constructed using the *prince.plot* function in the swamp package in R. The function generates principal components that explain part of the variation in the protein data set and then tests each variable against these components to evaluate possible associations. Heatmaps depict *p*-values (–log10-transformed) of these associations. Hierarchical clustering of the variables was generated using the *hclust* function.

Evaluation of Differentially Expressed Proteins

Longitudinal analysis was performed using linear mixed models and associations between occupational groups (welder and control) with serum proteins were fitted using the *lmer* function in the lme4 package in R. Variance explained by fixed factors (R^2_m) was calculated using *RsqGLM* function from the R package MuMin. The adjusted intraclass correlation coefficient was calculated using the *icc* function. The mixed models included study participants as random factors (random intercepts) and occupational group, age, and body-mass index (BMI) as fixed factors. Models were also constructed using additional variables such as physical activity, consumption of fish and vegetables, use of snus, and alcohol intake as random factors. Sensitivity analysis was performed on non-smokers only ($n = 236$).

Similar analysis using linear mixed model analysis was performed in welders after replacing the occupational group variable with respirable dust (in mg/m^3, adjusted for respiratory protection), years of welding (in years), or cumulative exposure. In this case, the linear mixed models included study participants as random factors (random intercepts) and age, BMI and respirable dust, years of welding, or cumulative exposure as fixed factors. To note that age and welding years are correlated ($r_S = 0.52$ for all welders included in this study, $n = 214$).

For the analysis of the cross-sectional group, we used multivariable-adjusted linear models (adjusted for age and BMI) to evaluate the associations between occupational groups (welder and controls) as well as measures of exposure (welding years, respirable dust, cumulative exposure) in welders with serum proteins.

RESULTS

Characteristics of the Study Participants

All study participants had a relatively healthy lifestyle: the majority were non-smokers, had low alcohol consumption and a medium-high intake of vegetables (**Table 1**). Compared with controls, welders were more likely to be born outside Sweden ($p < 0.005$) and to live in towns or countryside rather than in large or small cities ($p < 0.005$). There were no other significant differences between welders and controls. Age was significantly different between the study groups ($p < 0.001$); age for the cross-sectional group (one measurement) was more similar to the age for the longitudinal cohort (two measurements) at timepoint 1. There were no other significant differences between the longitudinal cohort and the cross-sectional group. BMI increased significantly at timepoint 2 compared with timepoint 1 in both welders and controls (**Table 1**). None of the other characteristics of the study participants changed significantly between the two timepoints.

As expected, both years of welding and cumulative exposure increased significantly for welders at timepoint 2 compared with timepoint 1 (**Table 1**). Median respirable dust in air, with and without adjustment for respiratory protection, was significantly higher at timepoint 1 (0.6 and 1.0 mg/m^3, respectively) compared with timepoint 2 (0.5 and 0.5 mg/m^3, respectively). The median stationary *area-level* of respirable dust concentrations in the control companies were 0.09 mg/m^3 (min-max: 0.02–0.2) for timepoint 1 and 0.03 mg/m^3 (min-max: 0.02–0.06) at timepoint 2. The cross-sectional group had median respirable dust levels of 0.8 and 1.6 mg/m^3, with and without adjustment for respiratory protection, respectively, which was higher than in the longitudinal cohort. Mn concentrations were measured in the respirable dust at timepoint 2 and the median concentration was 0.085 mg/m^3 (5–95 percentile: 0.003, 0.584 mg/m^3). The median adjusted respirable Mn concentration was 0.049 mg/m^3 (5–95 percentile: 0.003, 0.314 mg/m^3). Of those welders who worked unprotected ($n = 59$), 26 were exposed to levels of Mn dust above the OEL (0.05 mg/m^3) at timepoint 2. The correlation between Mn and respirable dust in air was $r_S = 0.90$ and the correlation after adjusting for respiratory protection was $r_S = 0.88$ (data at timepoint 2 evaluated, $n = 100$).

Variation of Neurology-Related Proteins in Serum

Principal component analysis (PCA) was performed to evaluate to which extent characteristics of the study participants could explain the variation in serum levels of neurology-related proteins. The complete description of the 87 measured proteins is appended in **Supplementary Table 2**. Cohort timepoints 1 and 2, and the cross-sectional group, showed similar patterns in the PCA heatmap with age and BMI being the most significant parameters explaining protein variation (**Figure 2**). For this reason, age and BMI were used as covariates in the statistical analyses to evaluate differential protein expression in relation to welding (see below).

In addition, we evaluated the intra-class coefficient (ICC) of linear mixed models where individuals were random factors and occupational group, age and BMI were fixed factors, and evaluated the contribution of each of these fixed factors to the protein variance. Approximately half of the proteins ($n = 45$) had a relatively low intra-individual variation (i.e., ICC> 0.6) out of which 6 had a low intra-individual variation (i.e., ICC> 0.8) (**Figure 3A**). The protein variance (R^2_m) explained by occupational group (welders or controls), age and BMI, is presented in **Figure 3B**. For 17 proteins, age explained more than 5% of the variation (5–27%) while for 11 proteins BMI explained more than 5% of the variation (5–16%). Occupational group explained more than 5% of the variation for 2 proteins TNFRSF21 (6%) and TMPRSS5 (5%). The proportion of protein variance explained by occupational group, age, and BMI together in a combined model varied between 1 and 30% for most of the proteins (for 5 proteins $R^2_m < 1\%$). The top 5 proteins most influenced by age were EDA2R, RGMA, RSPO1, MSR1, and ADAM23 (R^2_m 27–14%) while the top 5 proteins most influenced by BMI were KYNU, MSR1, CPM, N_CDase, and THY1 (R^2_m 16–9%).

The addition of extra fixed factors to the models (e.g., physical activity, consumption of fish and vegetables, use of snus and alcohol intake) did not have major impact on the ICC or R^2_m (ICC of the combined model >0.6 for 42 proteins, R^2_m of the combined model between 2 and 32%) (**Supplementary Figure 1**) compared to models only considering occupational group, age, and BMI.

Differential Protein Expression in Relation to Welding

Using a longitudinal analysis (linear mixed models) we identified 5 out of 87 serum proteins that were differently expressed in welders compared with controls (TNFRSF21, TMPRSS5, NEP, GDF8, and NMNAT1, **Table 2**, $p < 0.05$). None of the associations were significant after correcting for multiple testing. Sensitivity analysis performed in non-smokers at both timepoints, resulted in very similar effect estimates as the main analysis. In addition, IL12 and gal_8 also reached the significance threshold (p-value 0.045 and 0.040, respectively).

One of the proteins, nicotinamide/nicotinic acid mononucleotide adenyltransferase 1 (NMNAT1) was also differently expressed in the cross-sectional group (**Table 2**). The magnitude and direction of effect was similar between the longitudinal and cross-sectional analyses. Complete output data from the differential expression analysis between welders and controls is appended in **Supplementary Table 3**. Gene ontology terms (biological process) for NMNAT1 are included in **Supplementary Table 4**.

In order to investigate dose-response relationships, we evaluated longitudinal associations (welders only) between serum proteins and measurements of exposure to welding, i.e., respirable dust (adjusted for respiratory protection, $n = 42$ welders with two measurements), years of welding ($n = 56$ welders), and cumulative exposure ($n = 42$) (**Table 3**).

None of the five proteins (TNFRSF21, TMPRSS5, NEP, GDF8, and NMNAT1), differentially expressed in welders vs. controls, were associated with any measurement of exposure to

TABLE 1 | Characteristics of the longitudinal study group (cohort measured twice) and cross-sectional group (measured once) of welders and controls.

	Cohort timepoint 1		Cohort timepoint 2		Cross-sectional group		*p*-value[i]	*p*-value[°]
	Welders (*n* = 56)	**Controls (*n* = 67)**	**Welders (*n* = 56)**	**Controls (*n* = 67)**	**Welders (*n* = 102)**	**Controls (*n* = 89)**		
Continuous variables - median (5–95 percentile)								
Age (years)	44 (23–60)	44 (24–56)	50 (29–66)	50 (30–63)	44 (26–60)	44 (26–59)	-	-
Years welding	10 (1–28)	0 (0–12)	15 (4–34)	0 (0–12)	8 (2–29)	0 (0–4)	<0.001	-
Respirable dust (mg/m^3)[a]	1 (0.3–4.2)	-	0.5 (0.1–4.3)	-	1.6 (0.1–6.8)	-	0.030	
Respirable dust adjusted (mg/m^3)[b]	0.6 (0.2–4.2)	-	0.5 (0.1–1.8)	-	0.8 (0.1–3.4)	-	0.011	-
Cumulative exposure[c]	4.6 (0.4–35.3)	–	9.7 (1.9–36.8)	-	6.3 (0.7–33.6)	-	<0.001	-
Body-mass index (kg/m^2)	27.2 (21.9–31.4)	27.5 (22.3–34.4)	28.1 (22.4–33.2)	28.1 (22.0–35.6)	28.7 (23.3–37.6)	27.5 (23.1–32.3)	<0.001	0.019
Categorical variables[d] - *n* (%)[e]								
Country of birth (Sweden)	42 (75)	62 (93)	42 (75)	62 (93)	67 (66)	80 (91)	-	-
Education (university or higher)	2 (4)	7 (10)	2 (4)	9 (14)	9 (9)	10 (11)	0.946[j]	0.826[j]
Residence (large and small cities)[f]	11 (20)	34 (51)	10 (18)	29 (44)	22 (22)	43 (48)	0.956	0.611
Hobby exposure to particles[g]	13 (23)	11 (16)	12 (10)	14 (10)	29 (29)	14 (16)	1	0.513
Smoking history (ever smoked)	25 (45)	25 (37)	25 (45)	28 (42)	47 (47)	29 (33)	1	0.724
Smoking status (currently)								
Non-smoker	54 (96)	64 (96)	55 (98)	63 (95)	90 (89)	87 (98)	1	1
Party smoker	2 (4)	3 (4)	1 (2)	2 (3)	8 (8)	1 (1)		
Smoker	0 (0)	0 (0)	0 (0)	1 (2)	3 (3)	1 (1)		
Current snus use	16 (29)	12 (18)	16 (29)	11 (17)	32 (32)	21 (24)	1	1
Alcohol intake (≥3 times/week)	2 (4)	1 (1)	2 (4)	2 (3)	2 (2)	5 (6)	0.713[k]	0.714[k]
Vegetable intake (≥5 times/week)	36 (64)	43 (65)	32 (57)	50 (75)	59 (59)	57 (64)	0.684[l]	0.512[l]
Fish intake (at least once/week)	31 (55)	32 (48)	33 (59)	32 (48)	51 (50)	40 (45)	0.936[m]	0.427[m]
Physical activity (moderate/high)[h]	10 (18)	27 (40)	29 (52)	33 (50)	42 (42)	39 (44)	0.544[n]	0.715[n]

[a]Measured by personal sampling or estimated; [b]adjusted for personal respiratory protection equipment; [c]cumulative exposure was calculated from adjusted respirable dust data and reported welding year experience; [d]variables were categorized by "yes" and "no" unless otherwise stated; [e]percentage calculated relative to the total valid answers; [f]large and small cities as compared with towns and countryside; [g]exposure to welding fumes, dust, engine exhaust. or engine diesel during leisure activities; [h]physical activity that involves sweating at least once a week and for at least 30 min; [i]p-value for the differences between welders timepoint 1 and welders timepoint 2 calculated using paired samples Wilcoxon test for continuous variables and Fisher's exact test for categorical variables; [j]statistical test based on 5 categories for education from secondary school to university studies; [k]statistical test based on 6 categories for intake of alcohol from every day to never; [l]statistical test based on 8 categories from 3 per day or more to never; [m]statistical test based on 7 categories from once per day or more to never; [n]statistical test based on 4 categories from sedentary to intensive physical activity; [°]p-value for the differences between controls timepoint 1 and controls timepoint 2 calculated using paired samples Wilcoxon test for continuous variables and Fisher's exact test for categorical variables.

welding (**Supplementary Table 5**). The protein neuroblastoma 1, DAN family BMP antagonist (NBL1), was associated with respirable dust in the longitudinal analysis ($p = 0.036$) and non-significantly in the cross-sectional analysis ($p = 0.071$). We identified 5 proteins (GCSF, EFNA4, CTSS, CLM6, VWC2) significantly associated with years of welding both in the longitudinal and the cross-sectional analysis (**Table 3**). Gene ontology terms (biological process) related to these proteins are included in **Supplementary Table 4**. GCSF was the only protein significantly associated with years welding after multiple comparison adjustment (**Table 3**). None of the other proteins were associated with respirable dust or cumulative exposure in the cross-sectional analyses.

Differential Protein Expression in Relation to Other Factors

Finally, using the same statistical approach we evaluated the associations of serum proteins with age and BMI. These two variables were indicated by the PCA analysis to explain part of the variation in the serum proteins. Our results suggest that 38 serum proteins (18 after multiple comparison adjustment) were associated with age in linear mixed models adjusted for BMI (**Supplementary Table 6**). In addition, 19 serum proteins were also associated with age in the cross-sectional group (**Supplementary Table 4**). We identified 41 proteins (11 proteins after multiple comparison adjustment) that were associated with BMI in linear mixed models adjusted for

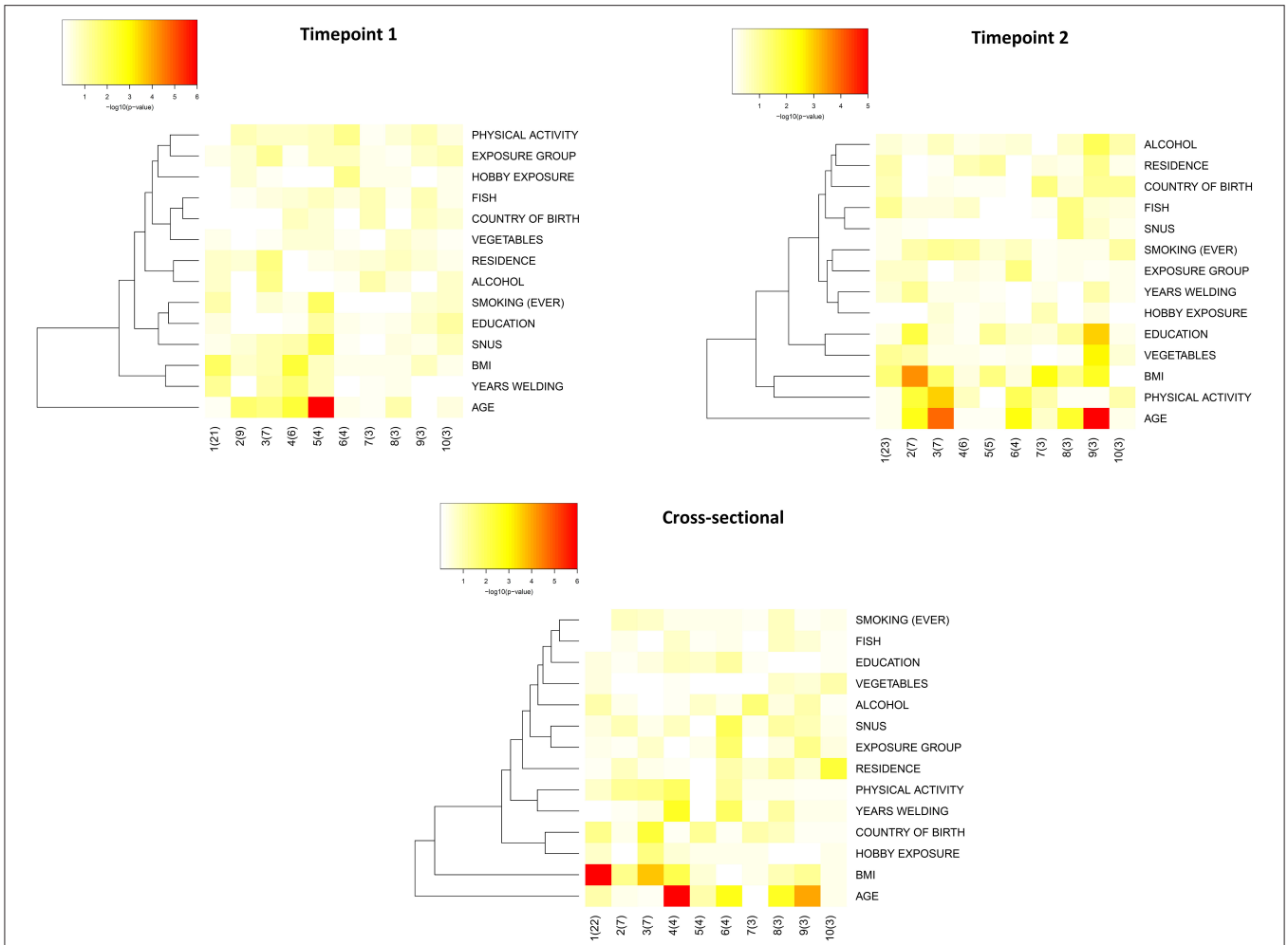

FIGURE 2 | Heatmaps of the principal components (PC) that explain the variation in the study groups. Heatmaps were constructed using input data from linear regression of association between the principal components of the data and the biological annotations. The influence of the biological annotations on the overall variation is plotted in a heatmap based on the *p*-value of the association. Input data was the normalized protein expression values (on a log2-scale). "group" refers to occupational group i.e., welders and controls; "residence" refers to current residence in large and small cities as compared with towns and countryside; "hobby exposure" exposure to welding fumes, dust, engine exhaust or engine diesel during leisure activities; "country of birth" is categorized as Sweden or outside Sweden; "education" is assigned to 5 categories for education from secondary school to university studies; "vegetables" frequency of intake of vegetables and is assigned to 8 categories from 3 per day or more to never; "fish" frequency is based on 7 categories from once per day or more to never; "physical activity" is based on 4 categories from sedentary to intensive physical activity; "ever smoking" stands for current or previous smoking and is categorized as "yes" and "no"; "alcohol" stands for frequency of alcohol intake and is stratified in 6 categories from every day to never.

age (**Supplementary Table 7**) of which 29 were significant in the cross-sectional group (**Supplementary Table 7**).

DISCUSSION

In this exploratory study, we investigated the influence of welding on levels of putative neurology-related serum proteins as a proxy of exposure-related changes in the nervous system. We found that welders exposed to respirable dust concentrations below the current Swedish OEL (2.5 mg/m^3) show change in serum levels of proteins related to neurological processes and neurologic disease. We do not know if the observed protein changes are related to exposure to Mn, particles or other factors present in the occupational environment of welders as we did not observe dose-response associations with different measures of exposure to welding fumes. In addition, we identified several proteins that are associated with age and BMI.

We found that being a welder was associated with higher serum levels of NMNAT1 in both longitudinal and cross-sectional analyses. NAD+/NADH are key players in redox reactions providing energy for metabolic reactions. Nicotinamide mononucleotide is converted into NAD+ by NMNAT that exists in 3 different isoforms. NMNAT1 is the nuclear form (26) and is widely expressed in all tissues, including the brain (www.proteinatlas.org). Further, NMNAT1 has a general role in neuronal maintenance, it prevents axon degeneration and has

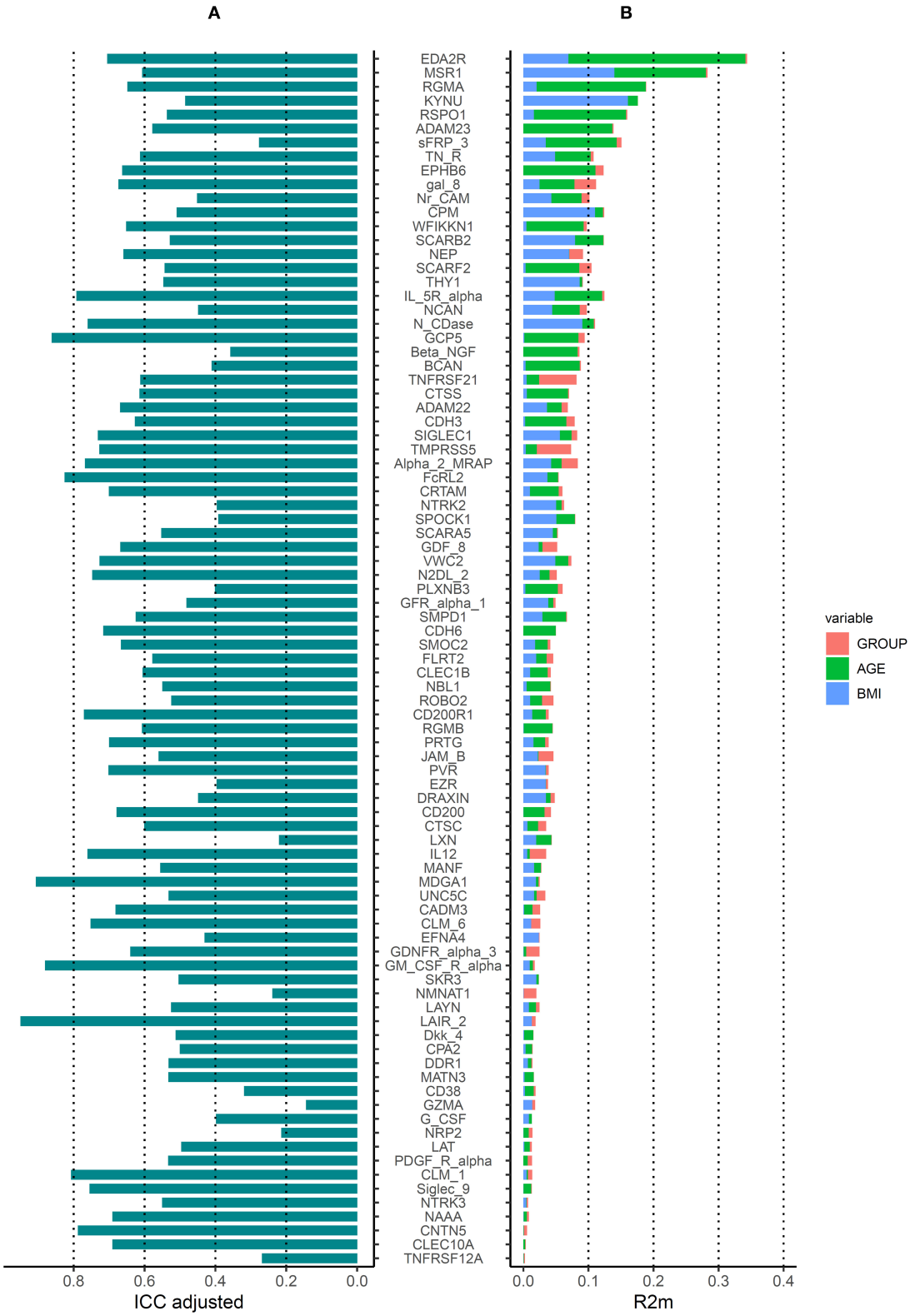

FIGURE 3 | Variation of serum protein from the Olink neurology panel. **(A)** Intraclass correlation coefficients (ICCs) estimated in linear mixed models including occupational group, age, BMI as fixed factors, and individual as random factors. **(B)** Protein variance explained (R^2_m) by occupational group, age and BMI generated from models including occupational group, age or BMI as fixed factors (one by one) and individual as random factors. Proteins are ordered according to the R^2_m of a linear mixed model including occupational group, age and BMI as fixed factors and individual as random factors.

TABLE 2 | Differentially expressed proteins in serum between welders and controls in the longitudinal study group (linear mixed models) and corresponding data for the cross-sectional group (linear models).

Protein	Linear Mixed Models ($n = 246$)			Linear models (cross-sectional group) ($n = 191$)		
	R^2_m (%)[a]	Beta (SE)[b]	p[c]	R^2 (%)[d]	Beta (SE)[e]	p[f]
TNFRSF21	8	−0.112 (0.039)	0.004	4	−0.008 (0.037)	0.838
TMPRSS5	7	−0.171 (0.059)	0.004	−1	−0.033 (0.056)	0.554
NEP	10	0.257 (0.116)	0.027	10	−0.022 (0.102)	0.833
GDF8	6	0.185 (0.087)	0.033	5	0.066 (0.072)	0.365
NMNAT1	2	0.256 (0.126)	0.043	7	0.28 (0.129)	0.032

SE, standard error; [a]Variance explained by fixed factors (group, age, body-mass index); [b]regression coefficient from linear mixed models interpreted as standard deviation difference in protein levels compared to controls, adjusted for age, body-mass index variables as fixed factors, and participant as random factors; [c]P-value from test of contribution of group inclusion (welders and controls) to protein variance using an analysis of variance approach with Satterthwaite approximation for degrees of freedom (Bonferroni-adjusted threshold for the p-value: $0.05/87 = 5.7*10^{-4}$); [d]variance in protein levels explained by the linear model; [e]regression coefficient from multivariable-adjusted linear models interpreted as standard deviation difference in protein levels compared to controls adjusted for age, body-mass index; [f]p-value from the linear model to test the difference between welders and control.

been shown to protect neurons from reactive oxygen species (26–28). Increased levels of NMNAT1 in welders may therefore reflect a protective response mechanism against the effects of long-term neurotoxic exposures, such as from oxidative stress (29) which has been associated with exposure to welding fumes (30). Studies in a mice model of Alzheimer disease have linked NMNAT1 to protection against Alzheimer disease (31, 32). There are, to our knowledge, no reports linking exposure to welding fumes to Alzheimer disease, and this needs to be explored in future studies. We would like to note that NMNAT1 is also associated with retinal function (33) and it is possible that the association found in our study could be related to exposure to UV radiation during welding. Exposure to UV radiation during welding has been classified as carcinogenic to humans (34).

In the dose-response analysis among welders only, associations with other serum proteins were found. Respirable dust was associated with increased expression of NBL1 (although not significant in the cross-sectional analysis), a protein widely expressed in the body and in blood expressed in T-cells (proteinatlas.org). NBL1 was initially identified as a tumor suppressor in a neuroblastoma cell line (35) and was later found important for nervous system and bone development (36). There is hitherto no relation described with NBL1 and neurodegenerative diseases or with manganese or iron. Together with the fact that the association with respirable dust was weak in the cross-sectional group, suggest that the findings for NBL1 should be interpreted cautiously.

In the dose-response analysis among welders only, we identified 5 proteins (GCSF, EFNA4, CTSS, CLM6, VWC2) that were associated with welding years, both in longitudinal and cross-sectional analyses. The respective proteins were negatively associated with welding years and apart from CTSS they were not associated with age. GCSF (granulocyte-colony stimulating factor) is a growth factor that has neuroprotective effects (37) and was shown to be expressed in lower levels in the brain of individuals with neurodegenerative diseases such as Parkinson disease and the rare degenerative disease multiple system atrophy (38). EFNA4 (EphrinA4) belongs to the ephrin family of proteins responsible for axonal guidance (39) and is involved in modulation of neuronal regeneration after injury (40). CTSS (cathepsin S) is a protease particularly expressed in the microglial cells of the central nervous system and *in vivo* studies in mice indicated CTSS upregulated with age and neuroinflammation (41, 42). CLM6 (also known as CD300c) is a receptor found on the surface of immune cells with yet an unclear function. VWC2 (also known as the brorin) is expressed both in neural tissues in embryos and in neurons in the adult mouse brain and is involved in neurogenesis (43). In a recent study plasma EFNA4, CLM6, and VWC2 were negatively associated with general fluid cognitive ability in old individuals, an association that appeared to be mediated by brain volume (44).

Although the protein changes identified cannot be clearly linked to Mn exposure of the welders, it should be noted that the Mn levels in the welders were high in relation to the current Swedish OEL, i.e., 0.05 mg/m^3. The median value of the adjusted respirable Mn concentrations was close to 0.05 mg/m^3 and half of the welders were assessed to be exposed to Mn concentrations above the OEL. Further, it should be noted that the measured unadjusted respirable Mn concentrations did not decrease compared with measured unadjusted concentrations reported in 2003–2005, i.e., 0.08 mg/m^3 (11). Thus, the respirable Mn exposure levels among welders in Sweden have not declined for the past 15 years although that the OEL has been reduced from 0.1 to 0.05 mg/m^3.

Being exposed above the OEL of Mn could increase the risk of adverse health effects on the nervous system in welders, as suggested from our study, and further protective measures should be taken. It is important that the general mechanical ventilation at the workplace is efficient and that local exhaust ventilation is available and used to reduce emissions at the source. Exposure can further be efficiently reduced by use of e.g., powered air purifying respirators, especially those with double visors.

This is an exploratory study, but we performed a rather strict analysis to identify proteins related to welding, by evaluating the proteins both in a longitudinal cohort and a cross-sectional

TABLE 3 | Differentially expressed proteins in serum in welders associated with exposure expressed as respirable dust (adjusted for personal respiratory protection equipment), years welding and cumulative exposure in the longitudinal study group (linear mixed models) and corresponding data for the cross-sectional group (linear models).

Protein	Linear mixed models			Linear models (cross-sectional group)		
	R^2_m (%)[a]	Beta (SE)[b]	p[c]	R^2 (%)[d]	Beta (SE)[e]	p[f]
Respirable dust (n = 84/96)						
KYNU	20	0.188 (0.063)	0.003	16	0.068 (0.046)	0.139
CTSC	12	0.099 (0.041)	0.016	8	0.045 (0.033)	0.172
GFRα1	12	0.061 (0.027)	0.027	10	−0.024 (0.021)	0.244
NBL1	10	0.034 (0.016)	0.036	2	0.025 (0.013)	0.071
Years welding (n = 112/101)						
GCSF	17	−0.025 (0.006)	<0.001[#]	7	−0.018 (0.008)	0.023
CRTAM	16	−0.022 (0.007)	0.002	1	−0.005 (0.007)	0.490
ADAM23	27	−0.023 (0.009)	0.011	−1	−0.009 (0.008)	0.227
IL12	9	−0.024 (0.01)	0.013	9	0.007 (0.008)	0.395
EFNA4	9	−0.008 (0.003)	0.014	22	−0.008 (0.003)	0.016
LAIR2	5	−0.033 (0.014)	0.017	1	−0.024 (0.016)	0.141
CTSS	17	−0.009 (0.004)	0.025	15	−0.006 (0.003)	0.042
CLM1	5	−0.017 (0.008)	0.028	4	−0.001 (0.008)	0.854
CLM6	6	−0.007 (0.003)	0.030	12	−0.007 (0.003)	0.033
VWC2	7	−0.013 (0.006)	0.047	25	−0.02 (0.006)	0.001
GFRα1	10	−0.009 (0.005)	0.049	12	−0.006 (0.004)	0.118
Cumulative exposure (n = 84/95)						
SCARB2	21	0.007 (0.002)	0.001	16	−0.001 (0.002)	0.635
LXN	14	0.004 (0.002)	0.008	4	0 (0.001)	0.820
PRTG	17	0.009 (0.003)	0.009	0	0 (0.002)	0.981
MDGA1	7	0.014 (0.006)	0.013	−1	−0.002 (0.004)	0.681
CD38	9	0.009 (0.004)	0.017	3	−0.002 (0.002)	0.243
GCP5	18	0.014 (0.006)	0.020	5	−0.002 (0.004)	0.621
FcRL2	16	0.011 (0.005)	0.024	1	0 (0.003)	0.988
sFRP_3	19	0.013 (0.007)	0.050	−2	−0.001 (0.003)	0.771

SE, standard error; [a]Variance explained by fixed factors (respirable dust/years welding/cumulative exposure, age, body-mass index); [b]regression coefficient from linear mixed models interpreted as standard deviation difference in protein levels per respirable dust unit increase/numbers of years welding/cumulative exposure unit increase, adjusted for age, body-mass index variables as fixed factors, and participant as random factors; [c]p-value from test of contribution of respirable dust/years welding/cumulative exposure to protein variance using an analysis of variance approach with Satterthwaite approximation for degrees of freedom (Bonferroni-adjusted threshold for the p-value: $0.05/87 = 5.7*10^{-4}$); [d]variance in protein levels explained by the linear model; [b]regression coefficient from linear mixed models interpreted as standard deviation difference in protein levels per respirable dust unit increase/numbers of years welding/cumulative exposure unit increase, adjusted for age and body-mass index variables; [f]p-value from the linear model to test the association with exposure variables (respirable dust/years welding/cumulative exposure); [#]significant after adjustment for multiple testing (Bonferroni).

group. It should be noted that changes in protein levels in this study were measured in serum and the relevance for processes in the brain should be interpreted cautiously as we do not know to which degree serum levels reflect expression levels in more relevant tissues. Therefore, to evaluate their relevance, it would be useful to measure the same proteins, e.g., NMNAT1, GCSF in other welding cohorts or in experimental studies of welding fumes. Proteins that were associated with welding in this study, if validated, could potentially function as biomarkers of neurotoxic effects of exposure to welding fumes. This is a longitudinal study, however, we acknowledge the limitation of only having two sampling points. The limited number of timepoints could overlook dynamic changes both in protein expression and exposure to welding fumes. A weakness with the study is that Mn exposure was not assessed at both timepoints, although we had a strong correlation between Mn and respirable dust at timepoint 2. Mn exposure is difficult to estimate by measurements in biological samples, partly due to highly efficient homeostatic mechanisms, and there is no validated biomarker for Mn exposure.

All in all, our study indicates that low-to-moderate exposure to welding fumes is associated with consistent changes in circulating levels of neurology-related proteins, that might be indicators of an increased risk for future disease. This highlights the need to further reduce the exposure levels to welding fumes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Ethic Committee Lund University, Sweden. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KW, MH, MA, and KB planned and designed the research and were involved in data interpretation. EA did the recruitment and medical examination of the study participants and data collection. MH did the exposure measurements. MH and TT did the exposure assessment. TL conducted the metal analysis. AG and TT conducted the statistical analysis. AG, KW, and KB wrote the manuscript. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00422/full#supplementary-material

Supplementary Figure 1 | Variation of serum protein from the Olink neurology panel. **(A)** Intraclass correlation coefficients (ICCs) estimated in linear mixed models including occupational group, age, BMI, physical activity, consumption of fish and vegetables, use of snus and alcohol intake as fixed factors, as well as individual as random factors. **(B)** Protein variance explained (R^2_m) by occupational group, age, and BMI generated from models including occupational group, age, BMI, physical activity, consumption of fish and vegetables, use of snus and alcohol intake as fixed factors (one by one) and individual as random factors. Proteins are ordered according to the R^2_m of a linear mixed model including occupational group, age, BMI, physical activity, consumption of fish and vegetables, use of snus and alcohol intake as fixed factors as well as individual as random factors.

Supplementary Table 1 | Metal concentrations in respirable dust measured among welders participating at timepoint 2.

Supplementary Table 2 | Normalized protein expression values. Data presented as median and interquartile range.

Supplementary Table 3 | Complete output of the differential protein expression analysis between welders and controls in the longitudinal study group (linear mixed models) and corresponding data for the cross-sectional group (linear models).

Supplementary Table 4 | Gene ontologies (biological process category) related to proteins that were differentially expressed in both the longitudinal and the cross-sectional analyses (according to uniprot.org).

Supplementary Table 5 | Associations of the significantly differentially expressed proteins in welders and controls with exposure to welding expressed as respirable dust (adjusted for personal respiratory protection equipment), years welding and cumulative exposure in welders only (linear mixed models).

Supplementary Table 6 | Proteins associated with age in the longitudinal study group (linear mixed models) and corresponding data for the cross-sectional group (linear models).

Supplementary Table 7 | Proteins associated with BMI in the longitudinal study group (linear mixed models) and corresponding data for the cross-sectional group (linear models).

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Opportunities and Challenges From Leading Trends in a Biomonitoring Project: Canadian Health Measures Survey 2007–2017

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Background: Biomonitoring can be conducted by assessing the levels of chemicals in human bodies and their surroundings, for example, as was done in the Canadian Health Measures Survey (CHMS). This study aims to report the leading increasing or decreasing biomarker trends and determine their significance.

Methods: We implemented a trend analysis for all variables from CHMS biomonitoring data cycles 1–5 conducted between 2007 and 2017. The associations between time and obesity were determined with linear regressions using the CHMS cycles and body mass index (BMI) as predictors.

Results: There were 997 unique biomarkers identified and 86 biomarkers with significant trends across cycles. Nine of the 10 leading biomarkers with the largest decreases were environmental chemicals. The levels of 1,2,3-trimethyl benzene, dodecane, palmitoleic acid, and o-xylene decreased by more than 60%. All of the 10 chemicals with the largest increases were environmental chemicals, and the levels of 1,2,4-trimethylbenzene, nonanal, and 4-methyl-2-pentanone increased by more than 200%. None of the 20 biomarkers with the largest increases or decreases between cycles were associated with BMI.

Conclusions: The CHMS provides the opportunity for researchers to determine associations between biomarkers and time or BMI. However, the unknown causes of trends with large magnitudes of increase or decrease and their unclear impact on Canadians' health present challenges. We recommend that the CHMS plan future cycles on leading trends and measure chemicals with both human and environmental samples.

Keywords: trend analysis, Canadian Health Measures Survey (CHMS), biomonitoring, time trend, body mass index (BMI)

BACKGROUND

The Canadian Health Measures Survey (CHMS) is a prominent and ongoing biomonitoring project that aims to assess the exposure and risk of environmental and non-environmental health hazards (1, 2). Both environmental chemicals in the air and water and biomarkers in blood, urine, and other bio-specimens have been obtained for quantification (3–6). Statistics Canada, the Public Health Agency of Canada, and Health Canada have been involved in the CHMS since its development before 2007 (7). The results from the CHMS help us to understand and track the levels of disease biomarkers and environmental chemicals, many of which have been considered health hazards by Health Canada (8).

One important feature of the CHMS is that it includes indoor air and tap water samples obtained at individual or household levels (1). The measurement using air and water samples enables researchers to derive nationally representative data on the levels of chemicals in their surroundings. Selected chemicals, particularly volatile organic compounds (VOCs) that include benzene and toluene, have been measured with both blood and air samples (6). In addition to the opportunities we expect in a biomonitoring project, measuring the same chemicals in blood and air samples illuminate the relationships between exposures and bio-accumulation with nationally representative data. Another opportunity for CHMS is its capacity to guide environmental and public health policies. CHMS has been used to monitor the disease burden of diabetes and hypertension in Canada (9, 10). Provincial or federal governments have enacted some policies to control exposure to environmental chemicals (11, 12). Results from the CHMS have the potential to demonstrate the effectiveness of these policies.

There are also challenges to the analysis of the CHMS data. Five reports have been published by Health Canada to describe the distribution of environmental chemicals in each of the five CHMS cycles (13–17). However, only basic statistics, including the mean, median and interquartile ranges, are reported for each biomarker (16). Occasionally, the statistics are stratified by age or sex, leading to insufficient sample sizes in certain subgroups (16). Without sufficient sample sizes, the statistics of the subgroups were not released to protect confidentiality (16).

Moreover, levels of environmental chemicals or biomarkers have not been considered for time trends. Trend analysis aims to illustrate the relationships between repeated measures (8). The time trends of many health measures have been regularly studied and updated using other biomonitoring data, especially those related to large disease burden and economic impact (18, 19). The time trend of childhood lead levels and secondhand smoke exposure have been studied to understand their respective impact on neurodevelopment and adverse health effects in the United States (20, 21). Another biomonitoring project, the German Environmental Specimen Bank (ESB), has explicitly described its objective as investigating the long-term trends of selected health hazards (22).

Abbreviations: CHMS, Canadian Health Measures Survey; BMI, body mass index.

In contrast, trend analysis has been used only for selected chemicals from the CHMS data, such as inorganic arsenic, triclosan, bisphenol-A, and phthalates (2). The levels of certain environmental chemicals in blood, such as lead, mercury, and arsenic, have been continuously monitored by Health Canada. However, the time trends were not released in the same reports (13–17). To facilitate the trend analysis of biomonitoring data, an efficient tool to implement trend analysis for national surveys has been published recently (7).

Another challenge to the analysis of the CHMS data is the adjustment of other confounders, particularly body mass index (BMI). Controlling for BMI is important for several reasons; BMI is associated with the distributional volume of biomarkers (23). Given the same amount of chemicals, larger distributional volumes can lead to lower concentrations in human bodies (24), and BMI is an important indicator of obesity. Obesity is becoming more prevalent and has emerged as one of the major issues in public health (8, 25). It is not clear whether obesity and BMI increases may play a role in the increases or decreases of biomarker trends. The measurement of environmental chemicals and biomarkers in CHMS cycles provides opportunities to quantify the potential impact of various health hazards and assess the burden of various diseases. We aim to address the challenges to biomonitoring data analysis by conducting trend analyses for environmental chemicals and biomarkers available in CHMS data while adjusting for the CHMS cycles, a proxy measure of time, and BMI.

METHODS

We used the biomonitoring data from CHMS cycles 1–5 (8). The five cycles were implemented between 2007 and 2009, 2009 and 2011, 2012 and 2013, 2014 and 2015, and 2016 and 2017 (8). Canadians who lived on reserves, those who were institutionalized, and full-time members of the Canadian Forces made up <4% of the total population and were excluded from the sample (8). More than 5,000 Canadians residing in 10 provinces aged 3–79 years were interviewed in each cycle (8). There were face-to-face interviews to record demographic and socioeconomic characteristics, as well as mobile clinic visits to retrieve blood and urine samples (8). Data on accelerometer-measured daily activities, lung function measured by a spirometer, medication, diagnosis of selected major disease, lifestyle, and health behavior, such as smoking and alcohol consumption, were also obtained (8).

Biomarker List and Variable Search

A comprehensive list of 447 unique biomarkers was provided in the CHMS cycles 1–8 Content Summary, 390 of which were available in cycles 1–5. There were 13 major themes in the biomarkers that are described in the Content Summary. These themes are allergies, bone health, cardiovascular health, chemistry panel, complete blood count, diabetes, environmental exposure, general characterization, infection markers, kidney health, nutritional status, reproductive hormones, and thyroid status (6). The environmental exposure biomarkers or environmental chemicals included measurements

that quantified the levels of chemicals in blood, urine, air, or tap water (5). Indoor air samples were determined at both personal and household levels (5). Non-environmental biomarkers were measured with either blood or urine samples in cycles 1–5.

The variable names of the biomarkers listed in the Content Summary (6) were not provided in the list. Therefore, it was necessary to match the biomarkers in the Content Summary list with the variables in the CHMS data dictionaries. To do this, all variable names were extracted from cycles 1–5 data dictionaries that were available from Statistics Canada. All CHMS variables available in the data dictionaries were screened to match the biomarker names in the Content Summary. In addition to the biomarkers listed in the Content Summary (6), the measures of vital signs including resting blood pressure (26), respiratory rates and heart rates were also included for analysis under the cardiovascular health theme (27). Among the 447 unique biomarkers or environmental chemicals in the Content Summary, 107 could not be linked to the CHMS variables. This led to the identification of 997 variables that were potential environmental or non-environmental biomarkers or environmental chemicals in the CHMS data sets, including administrative variables with names similar to the biomarkers or environmental chemicals, which only provided information on the limits of biomarker detection levels.

Variable Processing and Derived Variables

The trend analysis of all CHMS variables with and without adjusting for covariates was implemented. There were 54,235 variables in 85 data files from cycles 1–5 released before September 2019 (8). There were 16,727 variables in 35 files related to the use of bootstrap weights. The essential variables to control for survey design included sites, regions, CHMS cycles, and bootstrap weights. These variables were identified and introduced into the descriptive analysis and regression models. Unfortunately, there were discrepancies in the variable definitions across the CHMS cycles that needed to be resolved. For example, the levels of glucose and vitamin D were quantified with serum or plasma. Due to the limited differences between plasma and serum levels for respective biomarkers (8), the variables representing glucose or vitamin D levels were respectively unified. The glucose levels were measured among fasted individuals in cycles 3 and 4 and non-fasting subjects in cycles 1 and 2. To resolve these discrepancies, the glucose levels from cycles 3 and 4 were then labeled as fasting samples and analyzed separately from those from the non-fasting subjects in cycles 1 and 2. The levels of fibrinogen were measured with different units, g/cL in cycle 1 and dg/L in cycle 2, and to correct for this issue, they were converted to g/L.

Data Cleaning and Editing

The values representing "not applicable," "don't know," "not stated," and "not applicable" in all variables were recoded to missing (8). For certain measures, there were ranges of detection, and the levels of biomarkers or environmental chemicals were measurable only within these ranges. The ranges of detection might be documented in the data dictionaries or stored in separate variables. The values below the lower limits of the detection levels were replaced with half of the lower limits of detection levels, according to the imputation methods adopted by Health Canada (8, 13–16). The values representing measures higher than the upper limits of detection were replaced with 110% of the maximal levels of detection. Using the standard adopted by Health Canada, if more than 40% of the unweighted samples were found to have measures lower or higher than the limits of detection, this variable was not used for descriptive or trend analysis across cycles (8).

Descriptive Analysis and the Association With BMI

The basic statistics of all CHMS variables were gathered while controlling for the sampling frames and survey design (8). The statistics, including the mean and 95% confidence intervals (CIs), quartiles, and weighted sample sizes, were documented. For repeated measures, the rates of increase or decrease compared to the baseline were annualized based on the number of cycles since the first measures. For example, the levels of urine 2-hydroxychrysene, a type of chrysenes or environmental chemical, were measured in cycles 2 and 3. The levels in cycle 2 were used as the baseline. The annualized rate of increase or decrease was the geometric mean of the change rates between cycles (28). For example, the levels of biomarker A might increase up to 150% from cycles 1 to 4. The annualized increase rate was calculated as the cube root of 1.5.

For the continuous variables, the relationship with covariates was determined using multiple linear regressions while controlling for survey design (8). For binominal biomarker variables that only included two types of responses, their relationship with BMI was analyzed with multiple logistic regressions. In addition to BMI, there were other candidate predictors: sex, age, household income, education, CHMS cycles, use of over-the-counter drugs, and prescription use (8). The minimum and maximum ages of participants were 3 and 79 years, respectively (8). Household income was reported in Canadian dollars (8). Educational attainment included four categories that represented the highest levels of education that participants had attained: less than secondary school graduation, secondary school graduation, some post-secondary education, and post-secondary graduation (8). To avoid overlap with the trend analysis mentioned below, the CHMS cycles were converted to dummy variables. The first CHMS cycle, in which the dependent variables were measured, was used as the baseline. If the variables were repeatedly measured, the fixed effects of subsequent cycles were controlled for.

For certain variables, the predictors might not be used because only one of the categories of the predictors applied to the related outcomes in these cases. The predictors were dropped from the regression. For example, only males were eligible for a question asking if they had prostate cancer, and sex was not used as a predictor for the diagnosis of prostate cancer. For variables that were only applicable to school-aged children, education was not used as a predictor. The levels of total arsenic in blood were only measured in cycle 1, and the CHMS cycle was not

used as a predictor. Collinearity was assessed with variance inflation factors (29). If the squared variance inflation factors were greater than two, the predictors were documented and reviewed for collinearity.

There was a minimum sample size requirement for the regression analysis to control for survey design and to protect the confidentiality of the respondents (4). There should be a least one subject in each sampling site in each cycle. In total, there should be at least 100 observations for each cycle to meet the minimum sample size requirement.

Trend Analysis

Trends in CHMS variables were analyzed as the mean and 95% CIs across the CHMS cycles (8). The objective of the trend analysis was to understand whether there was an upward or downward trend across time or cycles for each variable (8). The CHMS cycles measured on a continuous scale were used as a proxy for time and the only predictor (8). If the variables were ever repeated and there were sufficient sample sizes for all cycles—the variables were eligible for trend analysis (8). The ratios of the repeated measures defined the upward or downward trend means compared to baseline levels, the first measurement in the CHMS. When the 95% CIs of the ratios in subsequent cycles included one, there were no significant increasing or decreasing trends.

Control for Survey Design

Stratified sampling was adopted in the CHMS and should be controlled for to obtain national estimates (8). To meet the requirement for degrees of freedom, the variables related to survey design were identified for all variables, including weights, CHMS cycles, interview sites, provinces of residence, and bootstrap weights (8). The results in this study are all weighted statistics. Two-tailed $p < 0.05$ were considered statistically significant. All statistical analyses were conducted with R (v 3.20) (30) and RStudio (v 0.98.1103) (31).

RESULTS

There were 997 biomarkers or environmental chemicals identified by matching the variables listed in the Content Summary and the Data Dictionaries. Population characteristics are listed in **Table 1**. There were more than 29 million Canadians represented in each of the five cycles, and the population numbers increased by cycle. About half of Canadians were female in the five cycles. Household income increased continuously. The mean age and BMI, respectively, decreased and increased across the CHMS cycles. On average, BMI increased at a rate of 1.002 per cycle.

In summary, 808 CHMS variables and 103 biomarkers or environmental chemicals repeatedly measured were significantly associated with the CHMS cycles on a continuous scale. Of all the variables, 1,147 variables and 167 biomarkers were significantly associated with BMI after controlling for the CHMS cycles, age, sex, household income, and education. If the use of prescription medication was also controlled, there were 440 variables and 53 biomarkers that were significantly associated with BMI. For biomarkers or environmental chemicals, there were 65, three, and one variable that increased by more than 10% compared to the levels measured one, two, and three cycles ago, respectively. There were 80, seven, and three biomarker variables that decreased by more than 10% compared to the levels measured one, two, and three cycles ago, respectively. Most of the biomarkers that increased or decreased at high rates were environmental chemicals.

To demonstrate the results, the leading trends of decrease and increase were plotted in **Figures 1**, **2**, compared to the trends of age in years and BMI in kg/m^2. Nine of the 10 variables that decreased the most rapidly were environmental chemicals. These variables are related to three chemicals and one biomarker in **Figure 1**: 1,2,3-trimethylbenzene (amount in ng or concentration in $\mu g/m^3$, air samples), dodecane (amount in ng or concentration in $\mu g/m^3$, air samples), palmitoleic acid (%, blood samples), and o-xylene (ng/ml, air samples). Only palmitoleic acid proportions (blood samples) decreased by more than 60% between cycles 2 and 3 and was not an environmental chemical. The amounts or concentrations of 1,2,3-trimethylbenzene, and dodecane were measured at the household and personal levels. The ratios of the four measures of 1,2,3-trimethylbenzene were all around 0.25 in cycle 3 compared with cycle 2, as shown in **Figure 1** (see **Appendix 1** for details). The ratios of the four measures of dodecane were around 0.37 in cycle 3, compared to cycle 2. The ratio of palmitoleic acid (in proportions) was 0.34 (95% CI = 0.29–0.40) in cycle 4, compared to cycle 3. O-xylene, a type of volatile organic compounds (32), was measured for the concentrations, and the ratio was 0.37 (95% CI = −0.003 to 0.75) in cycle 3, compared to cycle 2. Because most environmental chemicals were measured only in cycles 2 and 3, there was no information on these environmental chemicals in cycles 1 and 4. Palmitoleic acid (%) was considered a cardiovascular health marker (33) and the ratio was 0.32 (95% CI = 0.27–0.37) in cycle 4, compared with cycle 3. None of the levels of the variables in **Figure 1** were related to BMI in the regression models ($p > 0.05$ for all).

The variables, which increased the most rapidly, are shown in **Figure 2** (see **Appendix 2** for details). The 10 leading variables were all environmental chemicals: 1,2,4-trimethylbenzene (amount in ng or concentration in $\mu g/m^3$, air samples), nonanal (air samples), and 4-methyl-2-pentanone (amount in ng or concentration in $\mu g/m^3$, air samples). The ratios of 4-methyl-2-pentanone concentrations were 6.08 (95% CI = 2.18–9.97) and 6.13 (95% CI = 2.62–9.64) at personal and household levels respectively in cycle 3, compared to cycle 2. The ratios of 4-methyl-2-pentanone amounts were 5.29 (95% CI = 1.86–8.71) and 5.32 (95% CI = 2.12–8.51) at personal and household levels respectively in cycle 3, compared to cycle 2. The ratios of nonanal levels were 3.10 (95% CI = 2.05–4.15) and 3.04 (95% CI = 2.09–3.99) at personal and household levels, respectively, in cycle 3, compared to cycle 2. The ratios of the 1,2,4-trimethylbenzene concentrations or amount at the personal or household levels were between 2.55 and 2.89 in cycle 3, compared to cycle 2. None of the levels of the variables in **Figure 2** were significantly associated with BMI ($p > 0.05$ for all).

TABLE 1 | Characteristics of Canadians in the Canadian Health Measures Survey cycles 1–5.

Cycles	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Study time	2007–2009	2009 and 2011	2011–2013	2014–2015	2016–2017
Unweighted sample sizes (*n*)	5,604	6,395	5,785	5,794	5,786
Number of variables	6,798	6,971	9,709	7,686	2,928
Number of repeated measures	0	2,448	3,412	5,576	1,625
Number of biomarkers	327	925	864	327	143
Number of variables used to provide limits of detection or quantification (administrative)	0	168	176	0	0
Weighted *N* with full weights	29,235,444	31,026,646	31,663,898	32,275,596	32,255,596
Proportions of females	0.502	0.501	0.501	0.501	0.501
Mean ages (years)	39.3	38.6	39	39.3	39.4
Mean BMI	26	25.8	25.9	26.2	26.3
Mean household income (Canadian dollars)	77,818.5	80,085.7	84,779.2	92,165.8	93,065.4

BMI, body mass index.

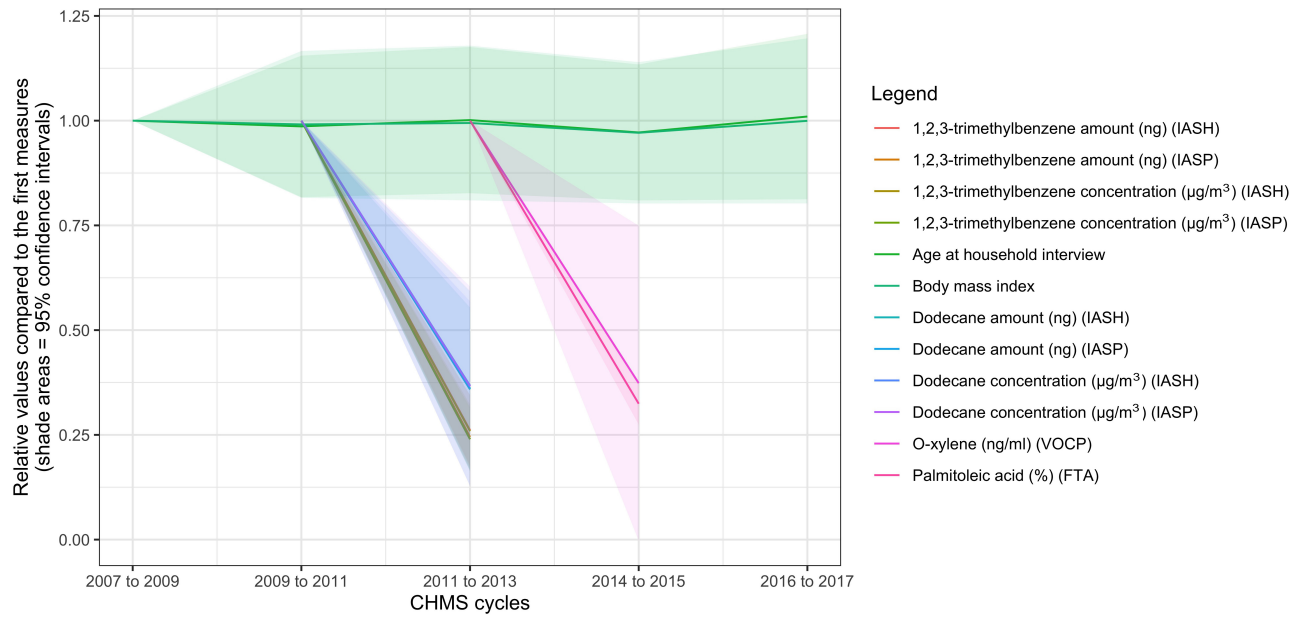

FIGURE 1 | Ten biomarkers with the highest rates of decrease in the biomonitoring data from the Canadian Health Measures Survey. FTA, red blood cell fatty acids subsample; IASH, indoor air household level; IASP, indoor air person level; VOCP, volatile organic compounds person level.

DISCUSSION

The CHMS biomonitoring project provides opportunities for researchers and policymakers to understand the exposure to health hazards and potential disease burden based on environmental chemicals and disease biomarkers, respectively. We have identified 997 biomarker-related variables in cycle 1–5, and there will be new biomarkers measured in subsequent cycles. However, there are also challenges to the analysis of the CHMS data, especially regarding the investigation of time trends and biomarkers' or environmental chemicals' associations with other confounders. First, a comprehensive study of all CHMS biomarkers is needed. If this is not possible, high-priority biomarkers or environmental chemicals for analysis need to be decided. The leading increasing or decreasing trends we identified have not been well-studied by researchers or CHMS administrators to our knowledge. Although there are a large number of variables collected in the CHMS, the number of variables in the CHMS remains manageable because efficient tools for trend analysis have been developed (8, 25). We urge the CHMS administrators to introduce these tools and release the latest trends of all environmental chemicals and biomarkers.

Second, the interpretation of the trends requires in-depth knowledge and multi-disciplinary expertise. The environmental and non-environmental biomarkers are distinctive and have different characteristics. Three environmental chemicals in this

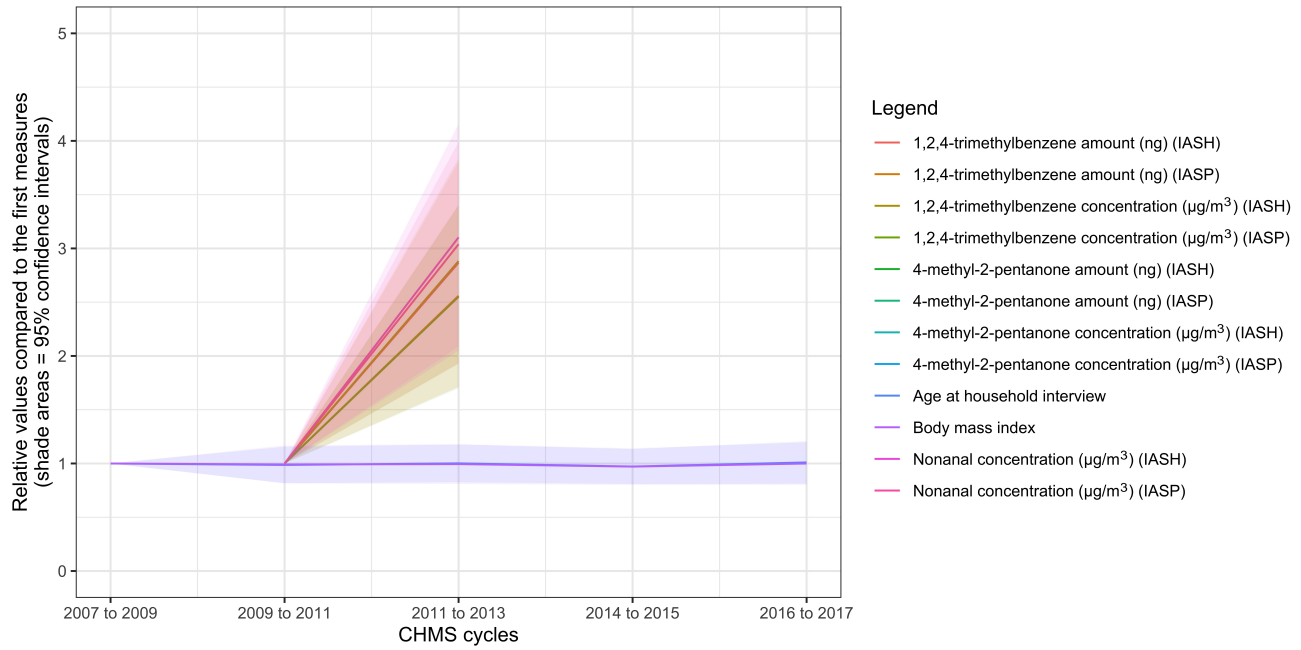

FIGURE 2 | Ten biomarkers with the highest rates of increase in the biomonitoring data from the Canadian Health Measures Survey. IASH, indoor air household level; IASP, indoor air person level.

study have the highest rates of decrease: 1,2,3-trimethylbenzene, dodecane, o-xylene (above measured with air samples), and palmitoleic acid (measured with blood samples). 1,2,3-trimethylbenzene can cause skin irritations and other symptoms (34). Dodecane can be used as a solvent for industrial use (35). O-xylene, a flammable oily liquid, is used to produce other chemicals or drugs (36). Palmitoleic acid is associated with metabolic risks in mixed directions (37).

Three environmental chemicals measured with air samples show the highest rates of increase: 1,2,4-trimethylbenzene, nonanal, and 4-methyl-2-pentanone. 1,2,4-trimethylbenzene is an important gasoline additive and is often used in the petroleum industry (38). Nonanal can be derived from nonanoic acid, and its metabolites have been observed in cancer metabolism (39). 4-methyl-2-pentanone, a type of colorless ketone, is an important industrial solvent (40). These chemicals and biomarkers are produced from different sources with varying degrees of health impact, although the potential sources and the overall impact remain unclear. Experts from several disciplines will be needed to further understand the sources and effects of these chemicals.

Third, there are other covariates to be investigated. We first focused on demographic characteristics and found that the role of BMI on the leading increasing or decreasing trends seems to be limited. The increase rate of BMI is relatively low, 0.2% per cycle on average (see **Appendix 1** for details), and many biomarkers increased at rates much higher than those of BMI. The role of diet and other factors related to exposure to environmental chemicals will need to be investigated.

Fourth, policy evaluation should be implemented immediately for these leading trends. Air quality has been monitored, and federal or provincial governments in Canada have revised standards (41). For example, Ontario, Canada's most populated province, has established air quality standards for 1,2,3-Trimethylbenzene and 1,2,4-Trimethylbenzene, two of the leading trends identified (11). In 2019, a risk management plan for 4-methyl-2-pentanone was proposed by the federal government (12). At this time, there has not been any progress published (12). Immediate action is required to assess the risks of these leading trends, and policy evaluation is warranted.

Lastly, the CHMS has the potential to be used to understand the relationships between exposure and accumulation in human bodies. However, only selected chemicals were measured with both blood and air samples for the same individuals, such as benzene in the CHMS cycle 2. None of the environmental chemicals with the leading trends we identified were measured with both blood and air samples in cycle 2 or 3. If the CHMS plans remain the same, there will be more biomarkers available for trend analysis and the comparison between exposure (air or water samples) and accumulation (blood samples) in the CHMS cycle 6 (6).

Recommendations for the CHMS Biomonitoring

After our extensive review of the codebook and dictionaries, we have several recommendations for the CHMS for biomonitoring. First, the identification of biomarkers in publicly accessible documents can be improved and made consistent. For example, several of the biomarkers or environmental chemicals listed in the content summary could not be retrieved in the data dictionaries. These biomarkers include 1-hydroxynaphthol and

2-hydroxynaphthol measured in urine, tribromomethane, and trichloromethane measured with air samples (6). Second, some basic clinical measures do not seem to be considered, such as blood osmolality and urine sodium that are important for kidney function evaluation (42). For the research community, it remains unclear why some measures were included while others were not. Our results show that biomarkers or environmental chemicals with large fluctuations are not necessarily reviewed or measured more frequently.

Third, the immediate implementation of a trend analysis of the CHMS data has several benefits. Trend analysis is a tool that can be used to identify some of the data errors (8, 25). Inconsistency in the measurement unit of fibrinogen, g/L and g/dL, is related to a 10-fold change that is much higher than the rate increases of any other variables and can be easily identified with trend analysis (personal communications with Statistics Canada). The identification of rapidly increasing or decreasing environmental chemicals is one of the first steps to assess the risks to human health. Understanding the significance of time trends is vital to plan for the biomarkers or environmental chemicals in subsequent biomonitoring. Currently, the biomarkers or environmental chemicals for biomonitoring have been decided up to CHMS cycle 8. The rapidly decreasing or increasing biomarkers identified in the study will not be measured in CHMS cycle 6 (6). The results of trend analysis may help prioritize the biomarkers or environmental chemicals for monitoring.

Lastly, the overall direction of CHMS can be better aligned to certain contexts. For example, if oriented to mimic the primary care settings, the CHMS can introduce other routine measures in primary care, such as body temperature and frequencies of common conditions, including common colds and influenza infections. Currently, there are more than 60 cardiovascular health variables conducted or planned for the CHMS. If oriented for the largest disease burden, cardiovascular disease (43), the CHMS can adopt measures in mean arterial pressure or even electrocardiography in the future.

Strengths and Limitations

The strengths of this study include the national representativeness of the sample, repeated measures of the majority of variables, well-structured data, and the interpretability of the results. However, there are several limitations to the trend analysis. First, data processing can be improved. Health Canada imputes the values below the limits lower than the detection limits by assigning half of the detection limits (14, 15, 17). There are other advanced methods available to impute censored information (44). Second, the programming codes are written at the Research Data Center under time and physical constraints and could be further streamlined for simplicity and execution efficiency. Four-day computation time may be further reduced (8). Third, the rapidly increasing or decreasing trends may be due to reasons other than the changes in their distributions or concentrations over time. Factors, such as sampling frames, non-response, sampling errors, measurement errors, and changes in measurement standards or methods, may play a role in the biomarker levels over time.

CONCLUSION

Trend analysis is a highly feasible method to screen all CHMS variables and can be used to select biomarkers increasing or decreasing rapidly across cycles. The associations with time and BMI are also possible to test. This helps to assess the potential health consequences related to the biomarkers or environmental chemicals and prioritize the biomarkers or environmental chemicals for investigation. We recommend the CHMS to plan future cycles based on the results of trend analysis, especially those increasing or decreasing at high rates. It is also possible to extend this trend analysis framework to other similar Statistics Canada data products, especially those with missing values coded in the same way as they are in the CHMS.

DATA AVAILABILITY STATEMENT

The datasets used for this study will not be made publicly available. It is against the Statistics Act of Canada to release the CHMS data or identify the individuals participating in the CHMS. Requests can be directed to Statistics Canada, statcan.maddli-damidd.statcan@canada.ca.

ETHICS STATEMENT

This secondary data analysis was approved by the ethics review committee at the Center Hospitalier de l'Université de Montréal. All methods were performed in accordance with the guidelines and regulations relevant to the analysis of public data. Written informed consent for the Canadian Health Measures Survey was obtained by Statistics Canada and not accessible to researchers.

CONSENT FOR PUBLICATION

Participants' consent for publication is not required for this data analysis project.

AUTHOR CONTRIBUTIONS

Y-SC conceptualized and designed this study, managed and analyzed data, and drafted the manuscript. C-JW assisted in data management and computation. H-CW, H-TH, L-CT, Y-PC, Y-CL, and W-CC participated in the design of this study. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00460/full#supplementary-material

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REVIEW
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Recent Advances in Arsenic Research: Significance of Differential Susceptibility and Sustainable Strategies for Mitigation

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Arsenic contamination in drinking water and associated adverse outcomes are one of the major health issues in more than 50 countries worldwide. The scenario is getting even more detrimental with increasing number of affected people and newer sites reported from all over the world. Apart from drinking water, the presence of arsenic has been found in various other dietary sources. Chronic arsenic toxicity affects multiple physiological systems and may cause malignancies leading to death. Exposed individuals, residing in the same area, developed differential dermatological lesion phenotypes and varied susceptibility toward various other arsenic-induced disease risk, even after consuming equivalent amount of arsenic from the similar source, over the same duration of time. Researches so far indicate that differential susceptibility plays an important role in arsenic-induced disease manifestation. In this comprehensive review, we have identified major population-based studies of the last 20 years, indicating possible causes of differential susceptibility emphasizing arsenic methylation capacity, variation in host genome (single nucleotide polymorphism), and individual epigenetic pattern (DNA methylation, histone modification, and miRNA expression). Holistic multidisciplinary strategies need to be implemented with few sustainable yet cost-effective solutions like alternative water source, treatment of arsenic-contaminated water, new adaptations in irrigation system, simple modifications in cooking strategy, and dietary supplementations to combat this menace. Our review focuses on the present perspectives of arsenic research with special emphasis on the probable causes of differential susceptibility toward chronic arsenic toxicity and sustainable remediation strategies.

Keywords: arsenic toxicity, differential susceptibility, arsenic methylation, single nucleotide polymorphism, epigenetic pattern, sustainable remediation

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INTRODUCTION

Arsenic exposure is one of the major threats to public health in more than 50 nations including China, Australia, India, Bangladesh, Argentina, Brazil, Thailand, Vietnam, Pakistan, Chile, Bulgaria, Canada, Czech Republic, Egypt, Iran, parts of USA, etc. (1). The worldwide scenario of arsenic contamination has been changing with the discovery of newer sites and increasing number

of affected people. The latest global count of arsenic-affected individuals, exposed above the WHO safety standard for drinking water of 10 μg/L (1), is ∼140 million, which has increased substantially over the decade (2). Since World War II, the initial reports of arsenic toxicity came up-front (3, 4) and have been a prime focus of environmental health research spanning various fields of research including geologists, chemists, pharmacologists, and more so biologists. Arsenic is a metalloid, its inorganic form (e.g., arsenic trioxide, sodium arsenite, and arsenic trichloride are trivalent forms, and lead arsenate and calcium arsenate are pentavalent forms) being found within the natural elements, while organic form circulates within the ecosystem. Common forms of organic arsenic compounds are methylarsonic acid, dimethylarsinic acid, arsanilic acid, etc., formed during metabolism inside living organisms in most of the cases (5). Inorganic arsenic is predominantly found in drinking water and dietary sources like dairy products, meats, cereals, etc. On the other hand, organic form like arsenobetaine is mostly present in seafood, fruits, and vegetables. Arsenic contamination in the groundwater of Indo-Gangetic region occurs from rapid weathering of arsenic-bearing rock in the upper Himalayan catchments, and various river systems get buried in young, low-lying alluvial floodplains of various riverine deltas. The elevated concentration depends on biogeochemical and hydrogeochemical process along with higher sedimentation rate. The slow aquifer-flushing rate is the primary reason for the higher sedimentation in these regions. A potential source of arsenic in the ecosystem is attributed to anthropogenic activities like mining, smelting, and industrial processes, use of arsenic-laden pesticides, etc. (2). Arsenic has three ionized states: arsines $As^{(III-)}$, arsenite $As^{(III+)}$, and arsenate $As^{(V+)}$, the latter two being most mobile in both organic and inorganic forms (2, 5). Both acute and chronic arsenic toxicities generate various deleterious effects in multiple organs and tissues, like hyperkeratosis and change in skin pigmentation, cardiovascular diseases, pulmonary disease, peripheral neuropathy, and developmental and cognitive impairments. Moreover, long-term arsenic exposure even at very low-level causes development of carcinogenic changes in the skin, liver, lung, bladder, and prostate (2). According to recent reports, chronic arsenic exposure around the WHO recommended level (10 μg/L) is also associated with increased risk of urinary tract cancer (6, 7). These evidence indicate that the current guideline for maximum permissible limit of arsenic in drinking water may still present a hazard to the population that are chronically exposed for a long time (8). Earlier researches on arsenic toxicity were primarily focused on population-based epidemiological outcomes, analysis of particular disease risk, chemical, and physiological aspects of arsenic metabolism, study of related gene expression profile, cancer, and DNA damage; all were associated with the mechanism of toxicity and subsequent outcome or disease manifestation. At present, the research perspective shifts toward the study of epigenetic alterations (DNA methylation, histone modification, and miRNA) to justify differential susceptibility toward arsenic exposure, detailed "omics" analysis (whole genome microarray, proteomic, and metabolomic profiling, etc.) of arsenic-induced cancer cases, bioremediation, and development of new therapeutic strategies, which are necessary for combating the outcome of arsenic toxicity as the affected population is increasing around the world. Since the year 2000, with advancement in high throughput techniques, arsenic research has evolved, and newer insights have been discovered (source: PubMed[1], **Figure 1**). Research trends from the year 2010 to 2019 indicate that "arsenic metabolism" remained the key focused area for arsenic research (**Figure 1**). It is indeed important to know the arsenic methylation status, which has been discussed in the following section to understand its toxic effects on biological system, related disease manifestation, and individual susceptibility. The next important focused area was found to be DNA damage and cancer. Understanding epigenetics was increasingly emphasized for the last 5–6 years, where the researchers attempted to explore the role of DNA methylation, histone modification, and miRNA alteration in arsenic toxicity as well as in arsenic-induced carcinogenesis. In this review, we try to string together the recent perspectives of arsenic research with special focus on understanding differential susceptibility in exposed population and how the innovative thinking may soon formulate better remedial strategies against this menace (**Figure 2**).

Abbreviations: As, arsenic; DMA^{III}, dimethylarsonous acid; DMA^{v}, dimethylarsonic acid; DNA, deoxyribonucleic acid; iAs, inorganic arsenic; miRNA, microRNA; MMA^{III}, monomethylarsonous acid; MMA^{v}, monomethylarsonic acid; RNA, ribonucleic acid; SAM, S-adenosyl-methionine; WHO, World Health Organization.

UNDERSTANDING THE PRESENT STATE OF RESEARCH

Sources of Arsenic Exposure

Arsenic is the 20th most abundant element found in the earth's crust, with an average concentration of 1–2 mg/kg in the continental crust (9). However, there are some geographical hotspots where the content is very high. It includes parts of South America and South and Southeast Asia, the latter two being most populated among all the regions and harboring nearly 75% of the total affected humans mentioned earlier. Arsenic is mobilized into the environment by naturally occurring processes like rainwater leaching, weathering, and seismic and volcanic activities. Another potent source of arsenic is through the emissions of arsenic-laden fumes and wastes that are carried by natural vectors like wind and water, expanding the topological periphery. To date exposure to arsenic is mostly due to groundwater contaminations where inorganic arsenic (iAs) normally exists in the form of arsenite/$As^{(III+)}$ or arsenate/$As^{(V+)}$. Depending on the oxidation potential of the microenvironment, the two states are interconvertible. The pH of the microenvironment regulates the ligand exchange process between the metallic oxides and hydroxides of iAs and the organic intermediates (microbiota) to release arsenic species (10). Higher concentration of arsenic tends to occur in association with metal oxides of iron as well as minerals

[1]https://www.ncbi.nlm.nih.gov/pubmed/ (accessed February 20, 2019).

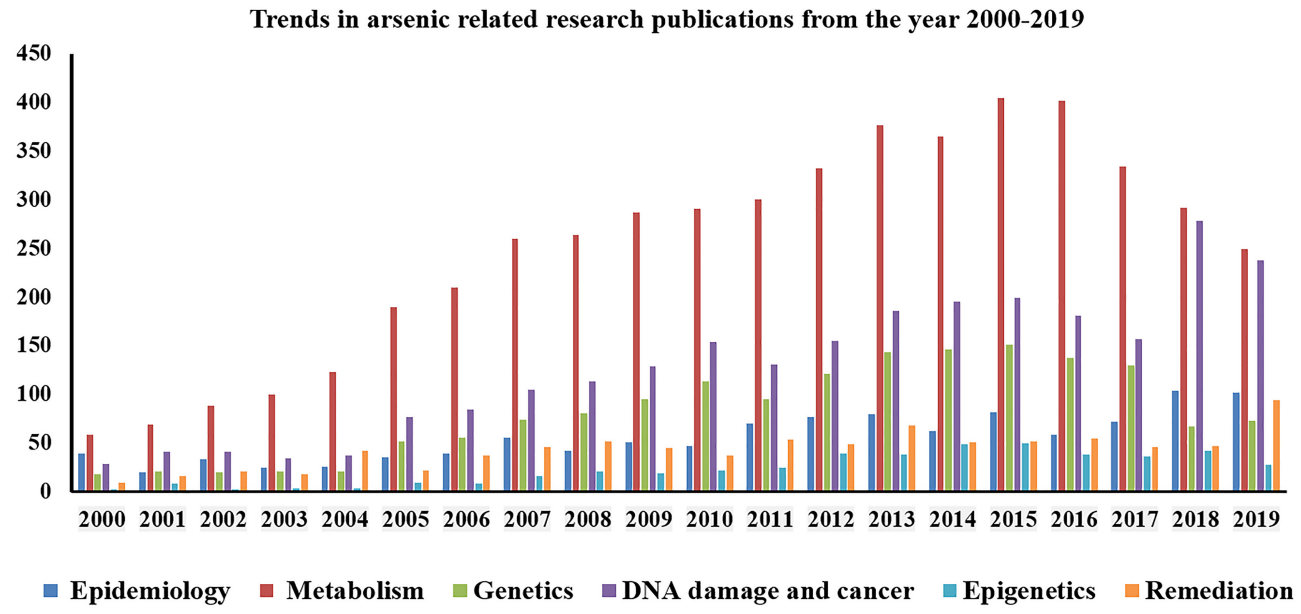

FIGURE 1 | Graphical representation showing trends in research publications on arsenic toxicity (from year 2000 to 2018).

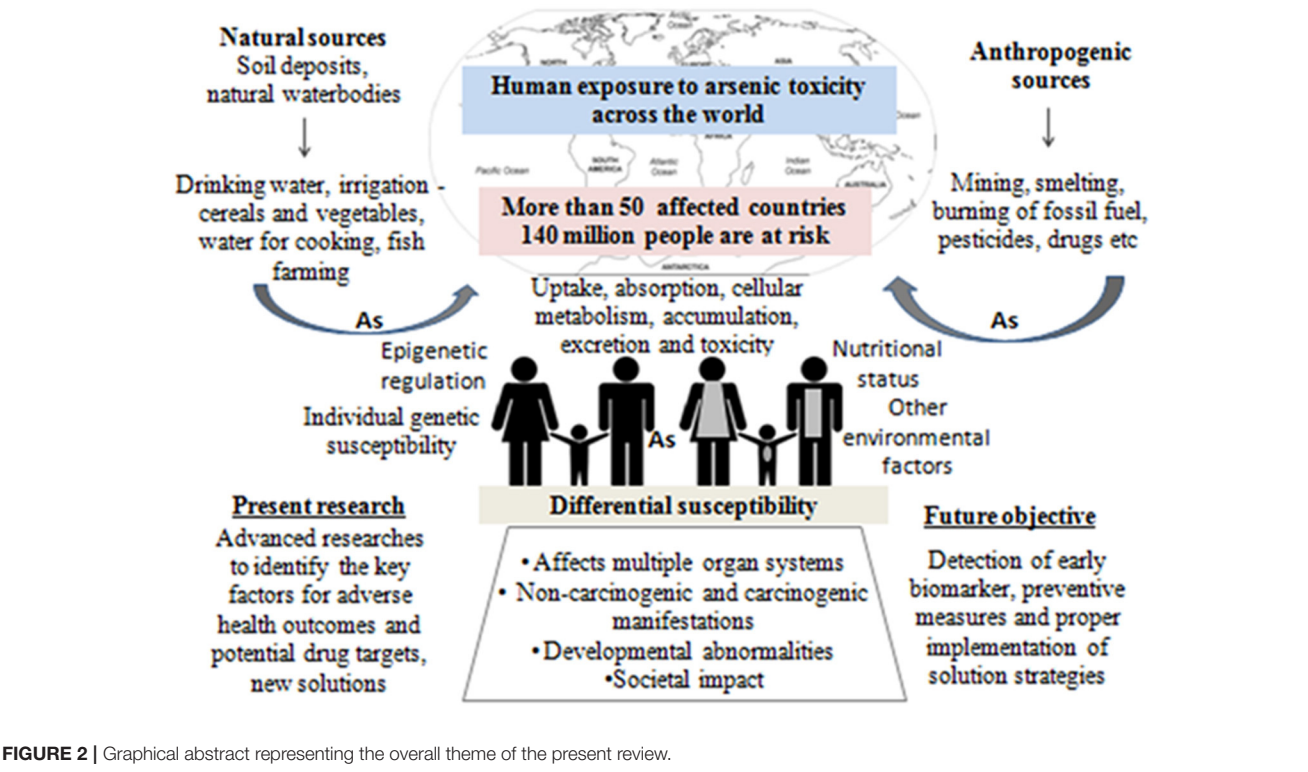

FIGURE 2 | Graphical abstract representing the overall theme of the present review.

with high sulfur content. In the Indian subcontinent, the Ganga–Brahmaputra–Meghna basin of the Indo-Bangladesh delta has high deposition of alluvial soil rich in sulfide drained down from the Chota Nagpur region. Increased weathering and rhythmic leaching of arsenic into the water table reflects that the increase in human intervention in abuse of chemical fertilizers laden with arsenic to aid agricultural yield have been associated with incidence of arsenic toxicity (11). Earlier, it was proposed that the organometallic component of arsenic in the groundwater might form complex and subsequently associate

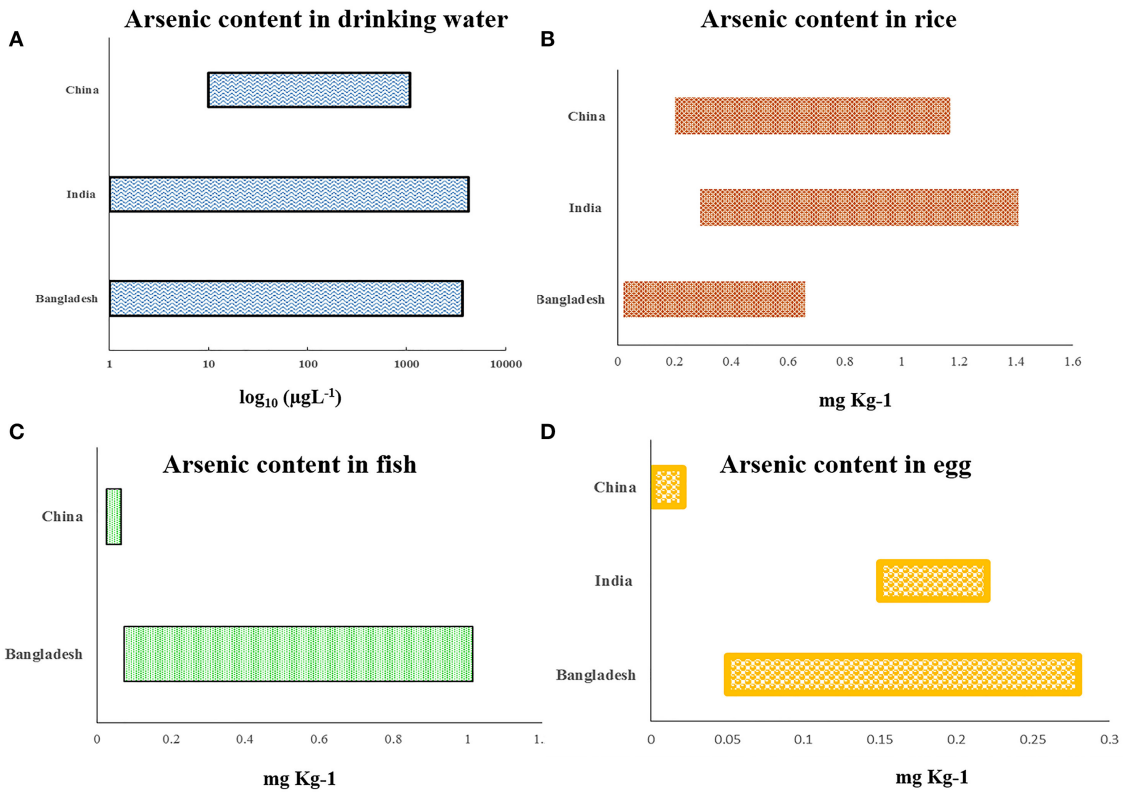

FIGURE 3 | Graphical representation showing recent country-wise reports about arsenic concentration found in **(A)** drinking water, **(B)** rice, **(C)** fish, and **(D)** egg (detailed report is in **Supplementary Table 1** with all references).

strongly with the dissolved arsenic anions, decreasing the release of arsenic, but research has shown that organic decomposition by certain bacteria generates anaerobic conditions whereby they release the arsenic species from these solid phases (12, 13). Apart from drinking water, relevant amount of arsenic exposure has been accounted for diet, especially through rice (**Figure 3**, **Supplementary Table 1**). The exposure becomes more pronounced in the region of Bangladesh and India, as the arsenic-affected zone have population whose staple is rice, grown and consumed locally (14). This shows that the dynamic spectrum of arsenic transports into the human physiological domain, the effects of which are being discussed in the following section.

Arsenic Metabolism: Toxic Nature Chemistry

Majority of the affected population are exposed to arsenic primarily through drinking water and food. Several studies have described the mechanism of arsenic metabolism inside human biological system (15–17). The inorganic form of oxy-anions, including pentavalent arsenite ($H_2AsO_4^-$) and trivalent arsenate (H_3AsO_3) is present abundantly in natural water. Organic form of arsenic is rarely found in the environment (18). The major metabolic pathways of iAs in humans includes several biochemical reactions like oxidation, reduction, methylation, thiolation, and glutathiolation (15, 19), of which methylation is critically important for the toxic pathology, tissue distribution, and cellular retention of arsenic. Arsenic in the form of arsenite/$As^{(III+)}$ or arsenate/$As^{(V+)}$ is absorbed in the gastrointestinal tract (GIT) of human, where arsenite is absorbed more rapidly than arsenate. $As^{(III+)}$ enters into the cell through aquaglyceroporins [AQP3, AQP7, AQP9, and AQP10], and $As^{(V+)}$ uses phosphate transporters, respectively, whereas cellular efflux of arsenic primarily occurs through ATP-binding cassette transporters (MRP1, MRP2, and MRP5). Apart from these, certain glucose transporters (GLUT1 and GLUT5) and organic anion transporting polypeptides were also reported to be responsible for cellular arsenic uptake under different circumstances (15). The initial reduction in arsenate to arsenite is done by liver enzyme arsenate reductase. It is then methylated by $arsenic^{(III)}$ methyl transferase (AS3MT) in the presence of S-adenosyl-methionine (SAM) as methyl donor to monomethylarsonic acid (MMA^v), which is reduced to monomethylarsonous acid (MMA^{III}). MMA^{III} is again methylated to dimethylarsinic acid (DMA^v), which is subsequently reduced to dimethylarsinous acid (DMA^{III}). The main metabolites monomethylarsonic acid (MMA^v) and dimethylarsinic acid (DMA^v), where arsenic is in the pentavalent state, are less toxic compared to inorganic arsenic (iAs) and readily excreted in urine. In humans, during metabolism, some arsenic is accumulated (about 40–60%) in skin, hair, nails, muscle, bones, and teeth as iAs and MMA (MMA^{III} and MMA^v), which may impart toxic effects in multiple

tissues and organs in their later life (16, 18, 19). Another pathway of arsenic metabolism shows sequential addition of methyl groups to trivalent arsenicals and its conjugation with glutathione generating intermediate derivatives like arsenic triglutathione (ATG), monomethyl arsenic diglutathione (MADG), and dimethyl arsenic glutathione (DMAG). A third pathway explained about the formation of iAs/protein conjugate and subsequent generation of methylated metabolites (17). Various studies have also shown effect of folate intake and folate metabolism on arsenic metabolism and related disease risks. The amount of folate intake and genetic variants of folate metabolizing enzymes might be responsible for interindividual variation in arsenic metabolism and differential disease susceptibility (19). Relative distribution of arsenic metabolites in urine is commonly used as a biomarker of current exposure and indicates the individualistic metabolism efficiency, which is one of the major causes of differential susceptibility (16, 20). The percentage of urinary metabolite varies between individuals; most of the literature suggests 10–30% iAs, 10–20% MMA, and 60–80% DMA (19, 20). Conventionally, concentration ratios of MMA/iA and DMA/MMA in the urine indicate the methylation capacity of the affected individual. In addition, the activity of AS3MT might have an association with the tissue-specific retention of various arsenic metabolites in the body and subsequently with individual susceptibility (21, 22). Very few reports are available on thio-DMA as urinary metabolite. Raml et al. (23) identified thio-DMA in the urine samples of Bangladeshi women, and also reported from *in vitro* studies that it was about 10-fold more cytotoxic than dimethylarsinate; however, the specific health consequences of such metabolites are not yet known (22). In another work by Taylor et al. (24), the urine samples from arsenic-contaminated seaweed consumers had been analyzed, and thio-DMA was detected as urinary metabolite. Future studies are required to identify and evaluate the possible outcome of such unique metabolites for better understanding of the toxic nature of this metalloid.

Physiological Manifestations of Arsenic Toxicity

The major hallmark of arsenic toxicity is the occurrence of dermatological lesions of various types like raindrop hypopigmentation, pigmentation, keratosis (palmer and plantar), and even skin cancers like basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and Bowen's disease. Interestingly, only 15–20% of the population show such manifestations (25, 26). Based on the toxic outcome on human health, arsenic is considered as Group I human carcinogen. In recent years, *in vitro* work with human cancer cell lines have helped to unravel various toxic mechanisms related to dermatological health outcomes and cancerous outcomes of the liver, lungs, bladder, and neuronal disorders, but most of the common health outcomes in human to date are dermatological lesions, peripheral neuropathy, liver damage, respiratory disorders, ocular inflammation and irritations, etc. (27–31). In the last decade, arsenic research at the cellular level revealed that arsenic alters the gene expression pattern within the cell and alters telomere length, epigenomic profile, cell cycle, etc. (32–36). One of the most explored aspects of arsenic toxicity in last decade has been on DNA damage and repair mechanism. Studies both in humans as well as cell lines have yielded results, which have made the detection of genetic damage as a reliable biomarker for arsenic-induced toxic outcomes (31, 37–39). Both chromosomal aberrations as well as micronucleus have been associated strongly with arsenic exposure and have shown prominent correlation to arsenic toxicity when compared with unexposed human subjects (40–42).

New Efforts Toward Understanding Differential Susceptibility

Arsenic-induced characteristic skin lesions have been considered for years as the hallmark of chronic toxicity. We have found that exposed individuals, residing in the same area, show varied dermatological lesion phenotypes even after consuming equivalent amount of arsenic over the same duration of time. In fact, our observation suggests that only a smaller percentage of the exposed individuals show arsenic-induced characteristic skin lesions (43). On the other hand, the precancerous lesions like plantar and palmer hyperkeratosis often lead toward detrimental malignancies in some of the affected individuals, whereas others retain only the precancerous forms lifelong. Different researches indicate several factors like individual arsenic methylation capacity, genetic susceptibility, epigenetic profile, etc. as major role players behind the differential susceptibility (16, 44).

Arsenic Methylation Capacity and Differential Susceptibility

The interindividual variation in arsenic methylation potential could be an important predictor of individual's susceptibility. Majority of the researchers found that people with skin lesions and high arsenic exposure are likely to have reduced arsenic methylation capacity with high trivalent species MMA in urine (45–48). The methylation capacity might reduce with increasing dose of arsenic, smoking and alcohol consumption, age, and nutritional folate deficiency (46, 49, 50). On the contrary, one study from China reported about higher methylation capacity among people above 40 years of age compared to below 40 years, but they also found positive correlation between %MMA and risk of skin lesion. On the other hand, women, especially at pregnancy, have increased methylation capacity than men and non-pregnant women, respectively, which may be due to the effect of estrogen, and children also have better methylation capacity than adults (51–53). A case–control study from China reported similar total arsenic concentration in skin lesion and no skin lesion group, but they found increased concentration of MMA among the skin lesion individuals (54), which indicates that efficiency of arsenic methylation is indeed important for differential susceptibility. A detailed account of recent studies regarding arsenic methylation efficiency and risk of skin lesion is summarized in **Table 1**. Arsenite methyltransferase (As3MT; EC: 2.1.1.137) catalyzes both monomethyl arsenate (MMA) as well as dimethyl arsenate (DMA) using SAM and arsenite and MMA as substrates, respectively. It oxidizes SAM to S-adenosyl L-homocysteine (SAH) in both cases. Recently, using

TABLE 1 | Summary of studies on individual arsenic methylation capacity and risk of skin lesions in chronic arsenic exposed population.

	Region and sample size	Source and analyte	Association with primary methylation index (PMI) and arsenic-induced skin lesion individuals (cases)	References
1	Taiwan, 52	Drinking water (urine)	Cases had higher percent of iAs and PMI than matched controls	(55)
2	Central Mexico,104	Drinking water (water and urine)	Cases had higher average MMA concentration compared to no skin lesions	(56)
4	Araihazar, Bangladesh, 1,635	Drinking water (water and urine)	The %MMA in urine and PMI were positively associated in cases, whereas SMI was inversely and % iAs was not associated	(57)
5	Pabna, Bangladesh, 1,200	Drinking water (water and urine)	A 10-fold increase in primary methylation ratio was associated with a 1.50-fold increased risk of skin lesions	(58)
6	China, 327	Drinking water (hair, water, and urine)	The relative proportion of MMA was positively related with skin lesion grade, SMI was negatively related with cases	(59)
7	Matlab, Dhaka, Bangladesh, 504	Drinking water (urine)	Cases had three times higher PMI	(60)
8	Matlab, Dhaka, Bangladesh, 1,030	Drinking water (water and urine)	Higher %MMA was found in cases	(61)
9	South of Shaanxi Province (China), 57	Coal combustion (urine)	Cases had higher urinary arsenic and lower SMI	(51)
10	Inner Mongolia, China, 31	Drinking water (blood)	High PMI and low SMI in cases	(62)
11	Yunnan province, China, 146	Arsenic smelting plant (water, urine)	Cases with increased percentage of MMA	(63)
12	Gansu Province, China, 155	Drinking water (urine)	Increased PMI, and reduced SMI in cases	(54)
13	Huhhot Basin, China, 302	Drinking water (water, urine)	Cases had higher levels of urinary iAs and MMA	(64)
14	Huhhot Basin, China, 302	Drinking water (urinary As)	Cases with higher urinary MMA%	(46)
15	Peoples republic of China, 548	Drinking water (water, urine)	Increased urinary MMA was associated to hyperkeratosis	(65)
16	Araihazar, Bangladesh, 4,794	Drinking water (water and urine)	MMA% was higher in skin lesions and DMA% was higher in without skin lesion group	(52)
17	Inner Mongolia, China, 207	Drinking water (water, urine)	Urinary MMA and iAs concentrations were positively associated with cases	(66)
18	Peoples republic of China, 479	Drinking water (water, urine)	Higher iAs and MMA was associated with cases	(45)
19	Shaanxi province, Inner Mongolia, China, 96	Coal combustion and drinking water (air, water, urine)	Subjects with skin lesions had higher urinary contents of iAs, MMA, and DMA	(67)
20	Pakistan, 398	Drinking water (water and urine)	Higher iAs% and MMA%, lower DMA%, indicating high PMI and low SMI among cases	(48)
21	Araihazar, Bangladesh, 1,464	Drinking water (water and urine)	Decreased urinary %DMA in cases	(68)
22	China, 119	Drinking water (water and urine)	Higher PMI in cases and higher SMI in patient recovery and improvement	(50)

As, arsenic; iAs, inorganic arsenic; MMA, monomethylated arsenic species; DMA, dimethylated arsenic species; PMI, primary methylation index; SMI, secondary methylation index.

X-ray crystallography, the molecular structure of *As3MT* in conjugation with As^{III} and SAH have been resolved (69). Several single nucleotide polymorphic (SNP) forms of *As3MT* have been identified in relation to arsenic. In the next section, we try to evaluate the structural aspects of *As3MT* polymorphisms along with SNPs in other relevant genes in relation to arsenic-induced toxic outcomes.

Single Nucleotide Polymorphism and Differential Susceptibility

Previous reports indicate that arsenic-induced health effects might be more deleterious among the exposed population carrying susceptible variants of genes primarily related to arsenic metabolism, oxidative stress, DNA damage repair pathways, etc. Variations in the gene for *AS3MT* have been shown to be the most influential parameter in urinary arsenic metabolites and different disease manifestations including carcinogenic outcome. Several population-based association studies were conducted with a number of SNP sites, among which G>A change in the C10orf32 region (rs 9527) was found to be associated with increased skin lesion risk in Indian (70) and Bangladesh population (71). *AS3MT*, Met287Thr polymorphisms (rs11191439) were reported to be having different arsenic methylation efficiency compared with the wild type and associated with risk of development of skin lesions, bladder cancer, and increased micronucleus frequency (72, 73). Apart from *AS3MT*, other important genes related to arsenic metabolism are Purine nucleoside phosphorylase (*PNP*), methylenetetrahydrofolate reductase (*MTHFR*), methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), cystathionine-beta-synthase (*CBS*), glutathione S-transferase omega 1 (*GSTO1*), and glutathione S-transferase omega 2 (*GSTO2*). *MTHFR* catalyzes the biochemical

conversion of 5,10-methylenetetrahydrofolate (5,10-methyl-THF) to 5-methyltetrahydrofolate (5-methyl-THF) during the formation of SAM, which acts as a methyl donor of arsenic methylation. *MTHFR,* C677T, and C1298A polymorphism was reported to be associated with increased urinary MMA%, decreased DMA%, and risk of arsenic-induced skin lesion in arsenic exposed population from Bangladesh, Argentina, and Taiwan (57, 74, 75). Chen et al. (76) observed that individuals with the *MTHFR* 677TT/1298AA and 677CT/1298AA genotypes were 1.66 and 1.77 times more susceptible to develop skin lesions, compared with those having 677CC/1298CC genotype. Catalyzing the reduction in As^{V} to As^{III} is one of the functions of PNP during arsenic metabolism. A case–control study on 428 subjects from arsenic-exposed region of West Bengal, India found that polymorphisms of *PNP*, His20His, Gly51Ser, and Pro57Pro were significantly associated with arsenic-induced skin lesions risk (77). Similar type of studies depicting association between *PNP* SNPs and arsenic-induced health effects were discussed in **Supplementary Table 2**. *CBS* catalyzes the conversion of homocysteine to cystathionine, which has an influence on arsenic methylation. Two studies on arsenic-exposed population from Argentina found C234709T and G4920037A variants of *CBS* to be associated with urinary MMA% (19, 78). However, to date, there are no reports from any other population regarding the *CBS* polymorphism, and thus, more studies are needed to confirm its association irrespective of ethnicity. *GSTO*s are another group of genes participating in arsenic metabolism. Several studies found significant association of *GSTO1* polymorphic variants with the risk of skin lesion and cancer. Previous studies reported that Ala140Asp was associated with urinary MMA% and skin cancer risk in population chronically exposed to arsenic from drinking water from Bangladesh, Taiwan, and China (54, 57, 74). However, studies on arsenic exposed population from India, Mexico, Hungary, Romania, Slovakia, and USA did not find any significant association of *GSTO1* polymorphism and arsenic-induced disease etiology (72, 77, 79, 80). Luo et al. (81) reported that, for *GSTO2,* AG genotype for rs156697 and the AG genotype or at least one G allele for rs2297235 had an increased risk of arsenic-induced skin lesions, and for *GSTO1*, individuals carrying at least one C allele for the rs11191979 polymorphism or at least one A allele or the AA genotype for rs2164624 or at least one A allele for rs4925 showed a significant risk of arsenic-induced skin lesions. Glutathione S-transferases (GSTs, including GSTM1, GSTT1, and GSTP1) are important protectors for arsenic-related oxidative stress. *GSTP1*, Ile105Val polymorphism was reported to be associated with skin lesion and urinary arsenic profile among arsenic-exposed population from Bangladesh (82), China (83), and Vietnam (22), whereas no association was reported from the population of India (43) and Turkey (84). Ghosh et al. (43) did not find any association of *GSTT1* null genotype with arsenic-induced skin lesion in a study on 422 Indian subjects but reported that GSTM1-positive genotypes are associated with a high risk of skin lesions. On the other hand, McCarty et al. (58) reported that wild-type *GSTT1* is associated with a higher risk of skin lesions than null genotype, but no association was found in case of *GSTM1*. In a recent study on 241 people from Italy, no association was found between arsenic exposure and urinary arsenic profile (85). Genetic variants in BER-pathway-associated genes such as 8-oxoguanine DNA glycosylase (*OGG*), X-ray and repair and cross-complementing groups 1 and 3 (*XRCC1*, *XRCC3*), and apurinic/apyrimidinic endonuclease (*APE1*) may alter the genotoxicity of arsenic. Multiple case–control studies reported about the association of polymorphic variants of these genes with arsenic-induced disease risk. A detailed description of most recent (considering last 10 years) population-based polymorphism studies stating population size, source of arsenic exposure, and polymorphic variant associated with the particular disease have been summarized in **Supplementary Table 2**. To date, several population-based studies revealed significant association between the genotypic variants and arsenic-induced disease manifestation; however, the exact mechanistic aspect behind the role of single nucleotide polymorphism of a specific gene in understanding differential susceptibility still remains questionable.

Epigenetic Alterations, Gene Expression, and Differential Susceptibility

Recent researches identified epigenetic regulations, which include primarily DNA methylation, histone modification, and miRNA interaction as one of the critical regulators of arsenic-induced disease manifestations. Dynamic reversibility of epigenetic marks is a truly significant property, and it may pave the pathway of epitherapeutics to overcome the road blocks in developing potential drug targets for curing diseases due to arsenic toxicity. Smeester et al. (86) did a comprehensive examination of DNA methylation levels within CpG islands for over 14,000 genes among arsenic exposed with skin lesion (arsenicosis cases) and without skin lesion individuals. They found 182 hypermethylated genes in arsenicosis cases, the majority of which is involved in cancer-associated pathways. A whole genome microarray-based study was conducted on Bangladesh population, where 10 subjects with newly developed skin lesion and 10 no skin lesion were selected from a previous cross-sectional study of 957 individuals to evaluate the possible epigenetic changes. Results indicated DNA methylation changes over time in people having arsenic-induced skin lesions compared to control. They found top 20 differentially methylated CpG sites of which 13 CpGs (*TCEB3B*, *CYC1*, *CDH4*, *RHBDF1*, *CCDC154*, *JAKMIP3*, *AGAP2*, *PL-5283*, *CHPF*, *PPAP2C*, *PCNT*, *SLC6A3*, and *MAP3K1*) were increased in % methylation, and 7 CpGs (*MYO3B*, *KIAA1683*, *LOC642597*, *C2orf81*, *ESRRG*, *PRDM9*, and *TNXB*) were decreased in % methylation between baseline and follow-up (87). Majumder et al. (88) observed a correlation pattern between different stages of arsenic-induced skin lesion and whole genome DNA methylation. A study on Bangladesh population showed that genomic hypomethylation of peripheral blood lymphocyte DNA is associated with 1.8-fold increase risk for skin lesions (89). Another genome-wide DNA methylation study on 120 individuals from China found changes in global DNA methylation among patients afflicted with arsenical skin lesions. They also depicted about detectable DNA methylation changes due to arsenic exposure over the generations even though exposure occurred decades ago. Chanda

TABLE 2 | Studies on arsenic induced alterations in promoter DNA methylation related to skin lesion status.

	Region and sample size	Source of arsenic (samples used for estimation)	Promoter methylation status of target genes	References
1	West Bengal, India, 158	Drinking water (water)	Hypermethylated promoter region of *p53* and *p21* in skin cancer patients	(95)
2	Murshidabad, West Bengal, India, 122	Drinking water (water, urine)	Significant hypermethylation in the promoters of both *DAPK* and *p16* genes in skin lesion cases compared to no skin lesions	(96)
3	Murshidabad, West Bengal, India, 245	Drinking water (water, urine)	Significant promoter hypomethylation of *ERCC2* gene with increased expression	(97)
4	China, 208	Coal combustion (hair, urine)	Promoter hypermethylation of $p15^{INK4b}$ in arsenical skin lesion group	(64)
5	Guizhou, China, 138	Coal combustion (hair)	Hypermethylation of *ERCC1* and *ERCC2* and suppressed gene expression were found with skin lesion arsenicosis patients	(98)
6	Poland, 111	Copper mill (urine)	Hypermethylation of *NRF2* and *KEAP1* and altered gene expression in occupationally exposed group	(92)
7	Southern Taiwan, 40	Drinking water (water)	Unmethylation at −56 and −54 bp CpG in the CCND1 promoter—a predictor for invasive progression in arsenic induced Bowen's disease patients	(99)
8	Murshidabad, West Bengal, India, 326	Drinking water (water, urine)	Significant promoter hypermethylation of *MLH1* and *MSH2* gene was observed in skin lesion individuals	(38)
9	Murshidabad, West Bengal, India, 390	Drinking water (water, urine)	Promoter hypomethylation and increased gene expression of *Tfam* and *PGC1α* in skin lesion and skin cancer patients	(94)

et al. (90) observed *GMDS* gene fragment hypermethylation in the peripheral blood leukocyte DNA of skin cancer persons exposed to arsenic and suggested as a biomarker for arsenic-induced cancer. *AS3MT* gene plays an important role in arsenic metabolism and its toxicological response. Gribble et al. (91) found marked promoter hypomethylation *AS3MT* gene in arsenic-exposed population from Arizona, but no reports are found regarding the relationship between skin lesion status and *AS3MT* promoter methylation to date. It will be an interesting and important finding for future researchers, which will help in mechanistic understanding of how epigenetic modification of *AS3MT* contributes in differential susceptibility. In another study by Janasik et al. (92), significant promoter hypermethylation of *NRF2* and *KEAP1* was observed among occupationally arsenic-exposed copper mill workers from Poland. Our group had reported about mitochondrial DNA hypomethylation among arsenic-exposed individuals from highly arsenic affected areas of West Bengal, India, but no significant difference between with and without skin lesion group was observed. However, mitochondrial DNA copy number was found to be significantly elevated among skin lesion individuals with increased expression of electron transport chain complex I, subunit *ND6* and *ND4* genes (93). Recently, we had reported about significant promoter hypomethylation with increased expression of mitochondrial biogenesis regulatory genes, *Tfam* and *PGC1α*, among arsenic-induced skin lesion individuals compared to no skin lesion group, chronically exposed to arsenic through drinking water (94). Few studies also reported about altered promoter methylation of various important genes and subsequent change in gene expression among individuals with skin lesion compared to those without skin lesion explaining the vital role of epigenetic changes behind differential susceptibility (**Table 2**). High-throughput whole genome omics studies are useful tools to analyze and identify specific gene expression alterations in response to arsenic toxicity. In a study of proteomic profiling of arsenic-induced keratosis samples, three key proteins were identified, which were consistently differentially expressed in lesional skin compared to unaffected skin. The cadherin-like transmembrane glycoprotein, desmoglein 1 (DSG1), was suppressed, whereas the expression of keratin 6c (KRT6C) and fatty acid binding protein 5 (FABP5) were significantly increased (100). Argos et al. (101) analyzed the effect of arsenic toxicity on the development of arsenical skin lesion status by genome-wide gene expression patterns, where the expression of about 22,000 transcripts was evaluated between with skin lesion and without skin lesion group. They found 468 differentially expressed genes between the two groups. The presence of genomic deletion(s) in a number of genes (*OR5J2*, *GOLGA6L7P*, *APBA2*, *GALNTL5*, *VN1R31P*, *PHKG1P2*, *SGCZ, ZNF658*) and long intergenic non-coding RNA (lincRNA) genes (RP11-76I14.1, CTC-535 M15.2, RP11-73B2.2) were associated with higher risk for development of skin lesions independent of gender, age, and arsenic exposure (102). In one study, HaCaT cell line was treated with low dose of arsenic (100 nM sodium arsenite) for 6 months, and then, SILAC-based quantitative proteomics approach resulted in the identification of 2,111 proteins, among which 42 proteins were found to be overexpressed and 54 downregulated upon chronic arsenic exposure (103). Altogether, these studies provide insight into molecular alteration behind differential susceptibility.

Another important regulator of epigenetic machinery is the different miRNAs, which may play vital roles behind arsenic-induced individual susceptibility. In a recent study, total miRNA expression analysis was done on premalignant and malignant skin lesion tissues (basal cell carcinoma and squamous cell carcinoma) from an Indian population chronically exposed to arsenic. A total of 35 miRNAs were reported to be differentially expressed among the three lesion types analyzed. Two miRNAs

(miR-425-5p and miR-433) were increased in both BCC and SCC relative to hyperkeratosis, indicating their association with malignancy. Two other miRNAs (miR-184 and miR-576-3p) were activated in SCC relative to both BCC and hyperkeratosis, suggesting selective induction in tumors capable of metastasis. Six miRNAs (miR-29c, miR-381, miR-452, miR-487b, miR-494, and miR-590-5p) were selectively suppressed in BCC relative to both SCC and hyperkeratosis (104). A previous study of Banerjee et al. (105) reported about 4.5-fold upregulation of miR21 in skin lesion individuals compared to the no skin lesion group. The expression of the downstream targets of miR21 (PTEN and PDCD4) varied inversely, but the expression of pAKT and PI3K varied proportionately with its expression levels. Another study on arsenic-treated HaCaT cell line identified differential expression of 30 miRNAs of which miR-21, miR-200a, and miR-141 might play a role in skin carcinogenesis (106). Very few studies are so far reported regarding this area, and thus, it is indeed an interesting field for present day researchers to enlighten with newer findings.

Quite a few studies showed arsenic-induced alteration in post-translational histone modifications (PTHM) including H3K36me2, H3K36me3, H3K79me2, H3K27me3, H3K9me2, H3K18ac, H3K9me2, H4K16ac, etc. (107, 108, 108–111)]. Most of them are either *in vitro* or in mouse models, but population-based studies justifying difference between with and without skin lesion individuals are really scarce. Cantone et al. (112) identified that increased H3K4me2 and H3K9ac is associated with inhaled arsenic particulate matter in steel plant workers. Two different studies on arsenic-exposed Bangladesh population observed alteration in H3K9me2, H3K9ac, H3K4me3, H3K27me3, H3K27ac, and H3K36me2 moieties (113, 114). Pournara et al. (115) reported decreased H3K9me3 and unaltered H3K9ac among a population from Argentina, chronically exposed to arsenic through drinking water. A study on Chinese population exposed to arsenic from indoor coal combustion reported that modifications of H3K18ac, H3K9me2, and H3K36me3 are associated with the degree of oxidative damage and the severity of arsenicosis. However, none of the studies particularly differentiate the degree of altered PTHM between arsenic-exposed skin lesion phenotypes compared to those with no skin lesion. Recently, our group has reported about two different PTHMs among chronic arsenic-exposed population from India considering the skin lesion status (38, 116). We identified significant upregulation of H3K79me1 in individuals with arsenic-induced skin lesion, and H3K79me1 was found to be regulated by the upstream methyltransferase DOT1L. Again, significant downregulation of H3K36me3 was found in the arsenic-exposed with skin lesion individuals with an impairment of mismatch repair pathway activated by arsenic-induced DNA damage. Cell-line-based *in vitro* studies and animal model are necessary for the identification of detailed molecular mechanism and for the observation of the outcome of advanced therapeutics (113). However, it is noteworthy to mention that only population-based studies of arsenic toxicity may confer greater opportunities in epitherapeutic drug development and identification of early biomarker for arsenicosis.

PLAUSIBLE MITIGATION STRATEGIES

One of the ready solutions of arsenic removal is the reduction at source and providing an alternative source of drinking water. Arsenic comes into our body mainly through drinking water sources and a variable dietary intake of food grown in contaminated areas (**Figure 3**, **Supplementary Table 1**). With more than 140 million individuals affected, it might not be possible to provide better infrastructure in the removal of arsenic for daily consumption of arsenic-safe diets. The huge economic burden due to the population size makes it difficult to implement the strategies. Though beneficial for the population, the cost of installation of new wells of 1,000 ft deep or more is prohibitive. Another major challenge is the maintenance of already installed filters. After few years of installation, these filters either did not work properly or gets saturated with arsenic.

Reduction in Arsenic at Source

This reduction can be done in two ways: (i) alternative arsenic-safe water source and (ii) treatment of arsenic-contaminated water. A comprehensive report is available with detailed pros and cons of the several methods (Wilsonweb, Water supply options[2]). Alternative sources include groundwater [deep tube well, shallow shrouded tube well (SST) and very shallow shrouded tube well (VSST), dug well, infiltration galley], surface water (protected ponds, pond sand filters, combined filters, household filters), and rain water harvesting. Apart from these alternative water sources, arsenic can also be removed from water by various treatments. For example, solar oxidation of arsenic kept in transparent bottles effectively reduce the arsenic load by increased oxidation from $As^{(III+)}$ to $As^{(V+)}$. The common rural practice of storing water for longer period of time in clear container reduces the arsenic concentration through sedimentation (**Figure 4**). Another effective way to remove arsenic is through coprecipitation and adsorption processes after reduction of $As^{(III+)}$ to $As^{(V+)}$ by bleaching powder (chlorine) or potassium permanganate. Aluminum alum, $Al_2(SO_4)_3.18H_2O$, ferric chloride, $FeCl_3$, and ferric sulfate, $Fe_2(SO_4)_3.7H_2O$, are some of the commonly used coagulants. This technology can be used in our daily households with some simple adaptations—popularly known as Bucket Treatment Unit (BTU) (Wilsonweb, Water supply options[2]). Additionally, various sorptive media columns with activated alumina, iron-coated sand, granular ferric hydroxide, etc. are commercially available. The most efficient way to remove arsenic is the use of membrane techniques like reverse osmosis, nano filtration, and electrodialysis, but these are highly expensive for mass scale installation.

Adaptation of Cooking and Irrigation Practices

Rice being the staple food for billions of people, it significantly contributes more arsenic than any other food source (16, 117). Irrigation with arsenic-contaminated groundwater poses another major threat to public health through food-chain contamination.

[2]http://wilsonweb.physics.harvard.edu/arsenic/conferences/Feroze_Ahmed/Sec_3.htm (accessed February 20, 2019).

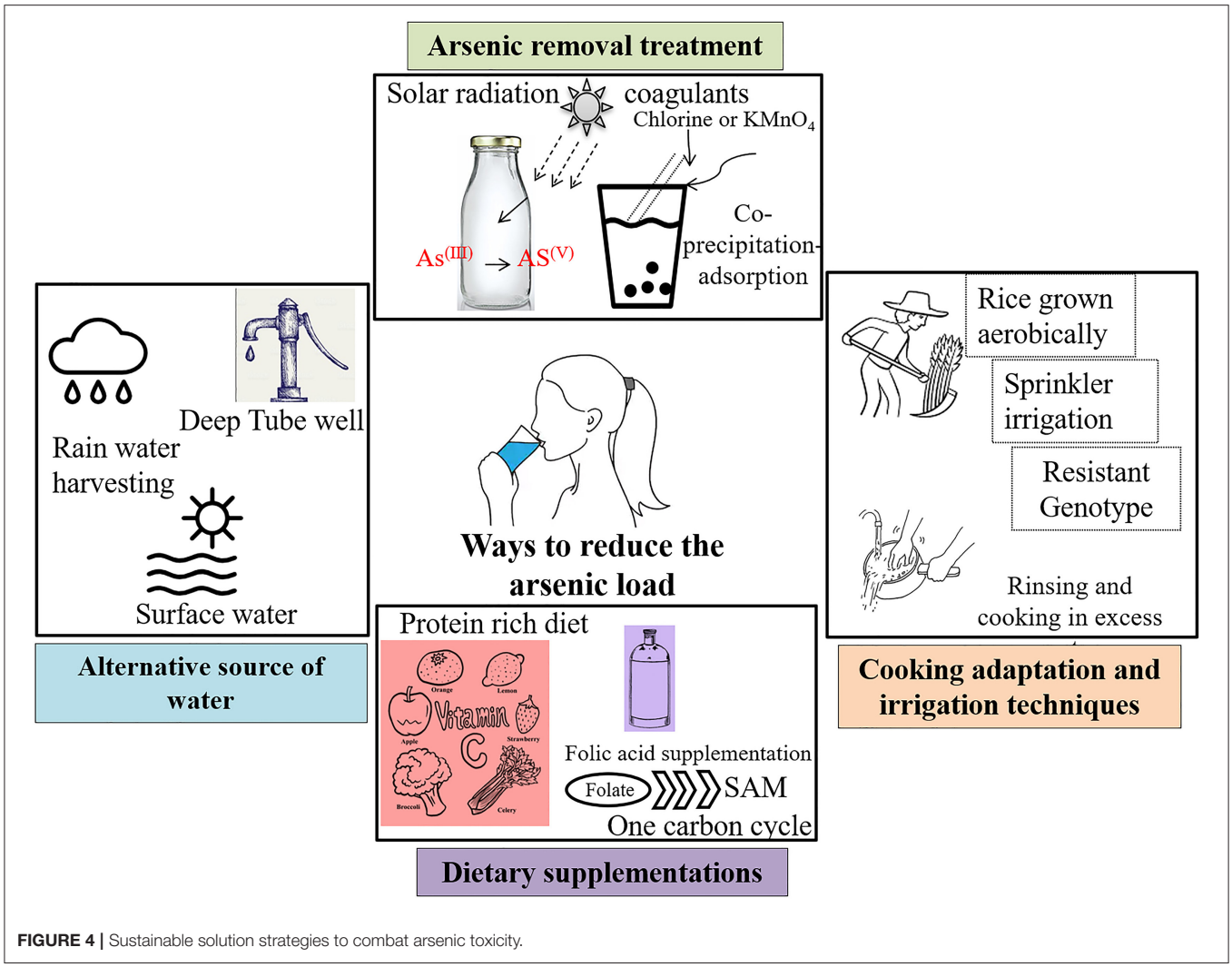

FIGURE 4 | Sustainable solution strategies to combat arsenic toxicity.

Conventional irrigation system in flooded paddies increases the arsenic uptake further (117). Recent studies have ascertained that the adaptation of different agronomic techniques can effectively reduce bioaccumulation in rice grains (118). For example, rice grown aerobically significantly has low arsenic accumulation compared to those grown in flooded condition (119). Again, another study reported the reduction in the total As during Sprinkler irrigation compared to traditional flooding (120). Few adaptations in cooking method can effectively lessen levels of arsenic within the grains. For example, a significant decrease was observed by thoroughly rinsing and then cooking the grains in an excessive amount of water, thereby increasing the percolation rate as arsenic is "mobile" in liquid water (117). A study in West Bengal showed that the traditional cooking method is most effective in lowering the concentration and the atab rice type with the lowest arsenic content (121). A recent review has discussed the possibility of genetically modified rice that would accumulate less arsenic within the rice grains (122). Very recently, rice varieties have been screened and developed that accumulate lesser arsenic, as consumption of arsenic-containing rice has been reported to cause genetic damage in humans (123–126). A recent review has discussed the possibility of genetically modified rice that would accumulate less arsenic within the rice grains (122).

Effects of Microbial Interactions

Microbiological interactions are crucial for the mobilization of arsenic into the aqueous phase leading to arsenicosis (127). Several arsenate-resistant microbes (ARMs) reduce $As^{(V)}$ to $As^{(III)}$ to thrive on the extreme conditions with high arsenic exposure. Recently, scientists have isolated a microorganism, which uses arsenic to "breathe," from Searles Lake with extremely unfavorable conditions (10 times saltier and 70 times more caustic, alkaline, pH 9.8) and loaded with toxic arsenic (~300 mg/L total arsenic) (Toxic Substances Hydrology Program, The U.S. Geological Survey[3]). These organisms could be used as a way of arsenic removal from water. Later, the applicability of the study remains undetermined (128). Water-usage switch

[3]https://toxics.usgs.gov/ (accessed February 20, 2019).

from surface to groundwater to reduce the risk of microbial contamination increased the risk of arsenic contamination. Two rod-shaped Gram-positive bacteria was reported to remove 51.45 and 51.99% of arsenite and 53.29 and 50.37% of arsenate, respectively, from arsenic-containing culture media isolated from Purbasthali block of Burdwan, West Bengal, India (129).

Dietary Supplementations

Studies have found that administering dietary supplements like vitamin C, different spectra of medicinal plants, drugs, etc. have reduced the effects of arsenic toxicity, although such observations have not been reported or worked out in humans (130–132). Vitamin C or ascorbic acid is a good antioxidant, which explains the reduction in oxidative stress created by arsenic-induced generation of reactive oxygen species (ROS) and in turn ameliorates the ill effects of arsenic within the body. Activation of antioxidative defense system within the body may be better and quicker. Nrf2 pathway is one of the prime pathways for the activation of antioxidant enzymes in the body (133–135). Research has shown that tea polyphenol brings about systemic activation of the Nrf2 pathway (136–138). We discussed in earlier sections that arsenic exposure depletes the methylation pool within the system. Studies suggest that administration of SAM reverses and/or reduces the degree of arsenic-induced DNA damage and anomalies *in vitro* (126, 139). Hence, a concoction of dietary supplements and reduction in arsenic intake can lead to better lives of the population affected by arsenic (**Figure 4**).

DISCUSSION

Interindividual susceptibility plays a key role in arsenic toxicity, which is already evident from the multiple research outcomes. Other than the variation in arsenic biotransformation, host-specific genetic make-up, and epigenetic regulation, an array of other environmental and physiological factors including nutritional status, lifestyle, effect of any other inherited or sporadic disease stage, and coexposure toward other heavy metals may regulate the extent of arsenic-induced skin lesion. Differential rate of arsenic methylation has been reported in many studies, where primarily a correlative outcome suggests that a higher primary methylation index is related to risk of skin lesion (*Reduction in Arsenic at Source*, **Table 1**). However, the complex mechanism of biotransformation behind skin lesion manifestation still remains elusive. Association studies on SNP and arsenic exposure in different populations have identified risk variants for developing skin lesion, several types of cancer, and other adverse diseases (*Adaptation of Cooking and Irrigation Practices*, **Supplementary Table 2**). However, there is inconsistency in the results because of population from different geographic areas, ethnicity, source of exposure, sample size, etc. Some studies are also limited on a single population and thus needed further validation [**Supplementary Table 2**, e.g., arsenic-metabolism-related genes *PEMT* and *DHFR* SNPs were validated only in Bangladesh population (140); similarly *CBS* SNP was analyzed only in Argentina population by (19, 78); in case of *DNMTs*, *Adiponectin,* and *INPP5A* genes single reports were found to date by Seow et al. (140–142), respectively]. The role of epigenetic modification to explain differential susceptibility is one of the most recent advances in arsenic research, having an interesting future prospect in the development of epitherapeutics. Besides the arsenic-related health effects, there are several other problems among the exposed population living in the arsenic-affected countries like India and Bangladesh, such as socioeconomic status, orthodox beliefs and stigma, lack of awareness, and poor maintenance of infrastructure. One of the best ways to counter arsenic-induced health problems is the intake of a protein-rich diet, as study showed that low intake of protein, choline, or methionine can reduce arsenic metabolism and excretion through urine. With a very low income, such a diet is not possible by the majority of the exposed population, leading to an ever-increasing number of susceptible individuals. It is suggested that citrus-based fruits with loads of ascorbic acid can also help in countering such a toxic effect (143, 144). Furthermore, different social stigma, superstitions, and prejudices about the skin symptoms further worsen the situation. In our own studies (38, 93, 116), while performing random sampling in such affected areas, we have faced discontent and discomfort among individuals and family members to acknowledge the fact of skin lesions like skin darkening, black spots, nodules, gangrene, and cancer in limbs, which causes social isolation of the victims. This stigma makes it difficult for our onboard dermatologists to determine whether an individual's dermatological lesion is due to arsenic or something else. While the public health communities and individual research organizations attempted to reach these populations, they refuse to interact with them, as no direct benefit could be offered toward them. Though years of research have been done, clinical implementation remained unsuccessful due to lack of risk–benefit analysis. Large population size is another major hindrance to implement any preventive measure like potable water supply to population at risk. Till now, no comprehensive therapeutic strategy has been tested. The natural means includes reduction in arsenic load in the drinking water, consuming protein-rich diet; ascorbates and polyphenols contained in regular beverages like tea seem to be the likely source of relief. In many parts of India and Bangladesh, the rural population fails to avail of arsenic-free water or high-protein diet due to lack of knowledge, financial restraints, and poor government infrastructure, and, on top of that, social taboo has kept the situation worse. Governments of developing countries cannot support the huge financial burden to set up arsenic-free deep tube wells or to distribute the arsenic removal domestic filter at individual scale. Even though, in few areas, arsenic filtration units have been installed, the durability of these units is less due to lack of filter replacement, poor monitoring, and delayed implementation. Therefore, the combating strategy is preferred to be bottom–up approach, where people will be aware of alternative and safe water sources along with suggested modification in cooking and irrigation practice (**Figure 1**). A comprehensive plan of action needs to be implemented, which should be disseminated among the affected people through awareness sessions.

CONCLUSION

The problem of arsenic poising is intertwined with several other factors like social beliefs, geological risk factors, poverty, and lack of awareness apart from its associated health risks that further worsen the scenario. Arsenic-induced dermatological anomalies often causes social isolation of affected individuals due to beliefs that arsenic poisoning is a "curse" or may be "contagious" in nature. Researches across the globe are trying to develop a sustainable solution, which is still missing. A holistic multidisciplinary approach is needed to combat with the menace. A lot of data have been generated, and several aspects have been explored. Research is now focused more on mechanistic detailing of individual risk of toxicity and developing strategies to counter arsenic toxicity both at the molecular and environmental perspectives. It needs a conjoint/consensus and a multidisciplinary approach to counter this environmental menace, a slow killer and showing an ever-increasing presence over the years.

AUTHOR CONTRIBUTIONS

TS had written the manuscript, composed figures, and tables. PB (2nd author) contributed in manuscript writing and figure composition. SP had contributed in manuscript writing. PB (4th author) had supervised the work and given necessary inputs. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.00464/full#supplementary-material

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Human Biomonitoring in the Oil Shale Industry Area in Estonia—Overview of Earlier Programmes and Future Perspectives

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Ida-Viru County, in Eastern Estonia, features industrially contaminated sites–where oil shale has been mined and used for electricity generation, and shale oil extraction. Higher prevalence of respiratory and cardiovascular disease has been found in the region due to high quantities of air pollution. Within the framework of "Studies of the health impact of the oil shale sector—SOHOS," this analysis aimed to map earlier human biomonitoring (HBM) studies and identify the suitable biomarkers for upcoming HBM in Estonia. Altogether, three studies have been conducted among residents: first, among adults in the 1980's; second, among children in the 1990's; and third, among employees, with a focus on workers and miners in the oil shale chemistry industry in the late 1990's and 2000's. In some of those studies, increased levels of biomarkers in blood and urine (heavy metals, 1-OHP) have appeared; nevertheless, in last 20 years, there has been no population-wide HBM in Estonia. According to air pollution monitoring and emission analysis, the pollutants of concern are benzene, PM_{10}, $PM_{2.5}$, and PAHs. In general, there is a decreasing trend in air pollutant levels, with the exception of a slight increase in 2018. One of the aims of HBM is to be analyzed if this trend can be identified in HBM, using similar biomarkers as applied earlier. The future perspective HBM could be divided into two Tiers. Tier 1 should focus on exposure biomarkers as heavy metals, PAH, and BTEX metabolites and Tier 2, in later stage, on effect biomarkers as Ox LDL, TBARS, etc.

Keywords: industrially affected lands, heavy metal, air pollution, PAH, HBM

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INTRODUCTION

In Estonia, mining, and use of mineral resource oil shale for generating electricity generation and producing shale oil, is concentrated in Ida-Viru County– a large-scale industrial area in Eastern-Estonia. Oil shale is a fine-grained sedimentary rock containing organic matter, in the form of kerogen, which yields substantial amounts of oil and combustible gas. Oil shale industry activities have led to various environmental problems in the region, the most important of which is ambient air pollution. Frequently, those industry affected areas are called industrially contaminated sites (1), defined as "areas hosting or having hosted human activities which have produced or might produce environmental contamination of soil, surface or groundwater, air, food-chain, resulting or being able to result in human health impacts" (2).

Ambient air quality affects health, as toxic substances entering the respiratory tract can also reach other organs through the bloodstream, and cause various health problems and diseases (3). Health effects, related to the oil shale sector, have been studied in Estonia; studies suggest poorer health indicators, including increased respiratory and cardiovascular disease prevalence and a decrease in life expectancy (4). In addition, children living in the area have a higher risk of developing asthma (5, 6). Also a recent study has found the lung cancer age-standardized incidence rate being higher in Ida-Viru County compared to Estonia overall (7). One of the weaknesses in epidemiological studies has been poor exposure data, as discussed by Orru et al. (4, 6). We believe the implementation of human biomonitoring (HBM), would help to specify data on exposures in the population and advantage to refine the results.

Within the framework of "Studies of the health impact of the oil shale sector—SOHOS," the current analysis aims to map the earlier HBM studies in the oil shale sector area, as well as elsewhere in Estonia, and identify the suitable biomarkers with an appropriate analysis methodology for the upcoming HBM program in Estonia.

HUMAN BIOMONITORING

The Human Biomonitoring Methodology

HBM is a methodology for assessing human exposure to natural and man-made compounds from living or working environments. Typically, specific substances or their degradation products, called metabolites, are measured in blood, urine, breast milk, and other body fluids or human tissues. The most commonly used and preferred biological matrix for HBM is blood, as it is in contact with organs and tissues where many chemicals are deposited (8, 9). However, there is a growing interest in non-invasive biomarkers, e.g., urine, that allow more routine sampling in human studies and reduce the number of blood sample refusers (9). Accordingly, previous studies of the health effects of the oil shale sector have used fractional exhaled nitric oxide (FeNO), which is a biomarker of airway inflammation (5, 6).

Compared to environmental monitoring, HBM has several advantages. For example, biological samples can characterize repeated exposure and the interaction of different exposures. HBM directly describes contaminants that have entered the body from all routes of exposure—inhalation, dermal absorption, and ingestion– and reflects individual differences due to different exposure levels, metabolism, and excretion rates. HBM data also refers to human physiological variations such as bioavailability, bioaccumulation, and persistence, which may increase the levels of some environmental chemicals (e.g., persistent organic pollutants) (9–11). Nevertheless, different matrixes (blood, urine, hair, breast milk, etc.) and different chemical physicochemical characteristics might characterize different exposures. If chemical substances, or their metabolites, are released rapidly from the human organism, repeated sampling at the individual level– to indicate a long-term exposure pattern– is needed (12).

The generally accepted classification of biomarkers divides them into two main categories: (1) exposure biomarkers and (2) effect biomarkers. Exposure biomarkers detect and measure chemical residues, or metabolites, in tissues or body fluids. It has been found that the advantage of utilizing exposure biomarkers is the so-called integrated measurement exposure, which is especially important in the case of substances with large differences in absorption depending on the time and location of exposure (9).

Effect biomarkers measure the processes that are considered to be "early events" associated with disease-related changes (13). An important group of effect biomarkers is genotoxicity biomarkers in workers or, to a lesser extent, in the population exposed to mutagens or genotoxic carcinogens. Many tests are used for the detection of DNA damage, such as micronucleus counting, Comet Assays (single cell gel electrophoresis), chromosomal aberrations, DNA adducts, etc (14).

However, effect biomarkers have been found to be effective only when people exposed to high levels of contaminants (e.g., working with mutagenic agents), and they are difficult to use to differentiate the effects of individuals. Toxicological studies have shown that individuals' responses to chemical exposure can often vary significantly (15). Ladeira and Viegas (14) consider that such differences between individuals may be genetically mediated or caused by some environmental stressor, disease process, or other epigenetic factor. Thus, effect biomarkers are currently recommended to be used as group indicators—they are sensitive, but not contaminant-specific and often difficult to interpret. Effect biomarkers have been used in earlier studies among oil shale sector workers (see chapter HBM Among Employees in the Oil Shale Sector for details).

National HBM Programs and HBM4EU Network

Many countries have established national HBM programs. The main objectives of these programs are to develop and validate biomarkers based on certain exposures and to predict the risk of disease for both populations and/or, under certain conditions, individuals (16). HBM can also identify spatial and temporal trends in human exposure as well as provide information on risk assessment. On the basis of risk assessments, decision-makers can be informed about chemical risks and policy measures can be initiated in order to protect population with the special focus on susceptible groups such as children and pregnant women (9, 11, 17). Successful examples of the impact of HBM include the banning of lead in petrol, avoiding mercury-containing amalgam teeth fillings, restricting the use of phthalates in plastics, and several other initiatives (18).

Within the Horizon2020 European HBM project HBM4EU has been established. It is now a joint project of 30 countries (Estonia joined in 2020), the European Environment Agency, and the European Commission for the period of 2017–2021 (19). The aim of this initiative is to promote and harmonize HBM activities in Europe, with the special focus to develop European HBM indicators (20). The harmonization of biomarkers collection and analysis– as well as the selection of which biomarkers to use– should make studies more comparable and give valuable reference material. Besides this, several other tools, such as

questionnaires, have been developed which can be used in further HBM studies, i.e., in an Estonian HBM program that includes oil shale area (19).

AIR QUALITY IN OIL SHALE INDUSTRY AREA

Previous studies have shown that the most important oil shale sector pollutants– that cause toxic and allergic reactions– are benzene, formaldehyde, phenol, particulate matter (PM_{10}), fine particles ($PM_{2.5}$), and polycyclic aromatic hydrocarbons (PAHs), including benzo(a)pyrene (B(a)P) (4–6). In Ida-Viru County, the air quality has been monitored in the cities of Kohtla-Järve, Narva, and Sillamäe, and in the Sinimäe region (**Figure 1**). In general, the air quality currently is said to be "good," with few exceedances of pollutants' limit values in recent years (21, 22). However, several studies have indicated that the health effects of PM_{10} and $PM_{2.5}$ also occur at concentrations below the set limit values (3), so health risks associated with air pollution still remain (4–6).

If we look at the long-term trends of pollutant concentrations, a slight decrease can be seen in the case of benzene in Narva, while in Kohtla-Järve the concentration has remained the same and in Sillamäe fluctuated from year to year (**Figure 1**). In the case of PM_{10} and $PM_{2.5}$, the concentrations have decreased somewhat over time, but spiked in 2018 (**Figure 1**). Concentrations of B(a)P have been on a slight upward trend in 2011–2014, then decreased sharply in 2015, and then started to increase again (**Figure 1**). In the analysis of concentrations of heavy metals and PAHs, bound to $PM_{2.5}$, we noted arsenic and cadmium remain at the same level in 2018–2019, whereas lead and nickel increased in 2018 in Narva and Kohtla-Järve, while both decreased again in 2019.

EARLIER HBM STUDIES IN ESTONIA

In our analysis, we conducted three larger HBM studies among residents and three among employees. The residents' studies were country-wide (incl. oil-shale area) with focus on adults and children; occupational studies focused on oil-shale sector employees.

HBM Among Residents

The largest HBM program took place in 1982–1985, where the content of heavy metals in the blood and hair of adults was studied in nine different regions in Estonia (**Table 1**). The study included residents aged 20–60 who had lived in the area for at least 5 years and were born, or lived, in Estonia for at least 20 years (23). In addition, later the data of residents from Maardu and Kostivere was analyzed (24).

The other two surveys were conducted among children in 1989–1990 and 1991–1994. In the first study (25), an increase in the concentration of some microelements in blood of oil shale region children was found according to the contamination of the external environment. The blood lead levels found in the children from Kohtla-Järve, Narva, and Sillamäe were on average three times higher than in Tartu (reference area). The cadmium concentration was 1.6 times higher in Kohtla-Järve compared to Tartu. The lead and cadmium levels in the blood of children from Kohtla-Järve were significantly higher compared to children from other two studied cities in Ida-Viru County. Compared to Tartu, the blood heavy metal concentrations were higher in all three Ida-Viru County cities. The second study (26) showed higher levels of lead and cadmium in children's hair in North-Eastern Estonia and higher levels of mercury in Western Estonia (in the hair of island and coastal residents). The higher levels of mercury are most likely related to the consumption of fish caught in the Baltic Sea. In that study, the values were also compared to limit values at that time. The lead values in blood were exceeded allowable limit of that time (27) in almost all regions, whereas cadmium levels were exceeded only in Kohtla-Järve and mercury levels in Western-Estonia and Lahemaa (affected by cement factory pollution).

HBM Among Employees in the Oil Shale Sector

The occupational studies have focused on miners and coke oven, and benzene plant workers. Due to weak ventilation systems and diesel-powered machinery, miners had a significantly higher exposure to benzene than oil shale sector's surface workers. In 2000, the concentration of benzene in the oil shale mine was 190 $\mu g/m^3$, while in the urban air of Kohtla-Järve the concentration of benzene was 29 $\mu g/m^3$ (28). Furthermore, mine workers are exposed to elevated levels of $PM_{2.5}$ and PAHs, which has resulted in 7.5 times higher levels of 1-nitropyrene (1-OHP) compared to ground workers (29, 30).

Of these studies, the first HBM looked at the exposure of coke oven workers to PAHs (31, 32). The workers were highly exposed, as the average amount of B(a)P in the air inhaled by workers was 5.7 $\mu g/m^3$ (32). The study also measured the urinary concentrations of 1-OHP and white blood cell DNA adducts. Though 1-OHP levels correlated with the number of DNA adducts, coke oven workers did not differ significantly compared to the controls from nearby Iisaku village (33). The subsequent analysis found that genotype might have played an important role regarding both biomarkers (1-OHP and DNA adducts), as evidenced by significant differences in number of adducts by genotypes among coke oven workers (34).

The second study compared benzene plant and coke oven workers. Higher benzene concentrations were observed at the benzene plant, which was confirmed with personal monitoring of benzene in the exhaled air (35). The same study also found higher levels of benzene in the workers' blood and elevated levels of the metabolite t,t-muconic acid (MA) in the workers' urine. The later analysis of effect biomarkers did not indicate an increase in the number of micronuclei in buccal cells (36) or increased cancer-specific ras (p21) proteins in plasma (37). A subsequent study of chromosomal aberrations did not show significant differences between the serum albumin levels of the controls from nearby countryside residents and the people working at the Kohtla-Järve chemical plant; nevertheless, the levels of S-phenylcysteine adducts were considered relatively high in both groups (38).

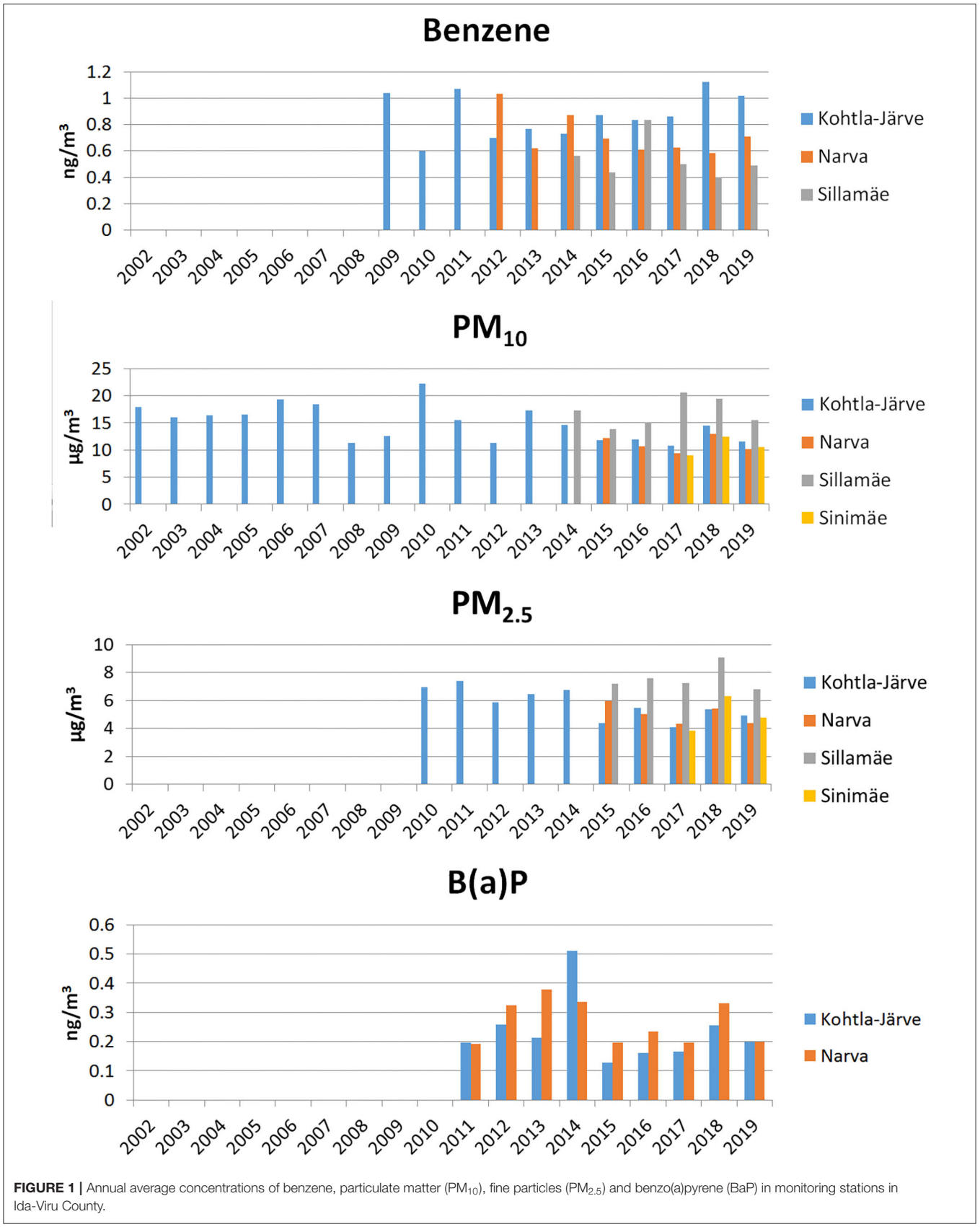

FIGURE 1 | Annual average concentrations of benzene, particulate matter (PM_{10}), fine particles ($PM_{2.5}$) and benzo(a)pyrene (BaP) in monitoring stations in Ida-Viru County.

TABLE 1 | Average content of heavy metals in blood (μg/100 ml) and hair (μg/g) [based on (23–26)].

	Lead			Cadmium			Mercury			Copper	Zinc
	Blood		Hair	Blood		Hair	Blood		Hair	Blood serum	Blood serum
	Adults	Children	Adults	Adults	Children	Adults	Adults	Children	Adults	Children	Children
Cities in oil shale region											
Kohtla-Järve	17.12[3]	–[2]	11.07	0.96[3]	–[1]	2.37	0.53[1]		0.77		
Sillamäe 1990/94*		9.0/5.5			0.54/0.18					80.2/59.9	156.2/130.4
Narva		–[2]			–[2]						
Country-side affected by cement production											
Lahemaa	22.23[3]		12.83	0.47[2]		1.08	0.83[3]		0.65		
Capital city											
Tallinn	8.25[3]	–[1]	8.67	0.48[2]	–[0]	1.30	0.37[1]		0.62		
Industrial complex near Tallinn											
Maardu	16.0[4]		13.1	0.80[2]							
Kostivere	13.3[3]	–[1]	10.4	0.68[1]							
Southern Estonia											
Tartu 1990/94*		4.5/ 2.5[1]			0.70/ 0.17[1]					65.0/ 90.9	163.3/157.8
Võru	7.41[3]	–[1]	8.58	0.34[2]	–[0]	0.82	0.36[1]		0.84		
Western Estonia and islands											
Saaremaa	13.87[3]		10.80	0.57[2]		1.33	0.65[2]		1.90		
Haapsalu	12.52[3]	–[0]	11.13	0.32[1]	–[0]	0.65	0.82[3]		3.09		
Hiiumaa	9.15[2]		7.24	0.23[2]		1.27	0.49[2]		2.30		
Matsalu	8.22[1]		2.20	0.24[1]		0.30	0.79[3]		2.52		
Viidumäe	9.46[2]		5.07	0.21[1]		0.54	0.46[2]		1.14		

[4] *>2 times of "limit value,"* [3] *1–2 times of "limit value,"* [2] *½–1 times of "limit value,"* [1] *½–¼ times of "limit value,"* [0] *<¼ times of "limit value,"* _0,1,2 *no exact value provided, only compared with "limit value".*

**Original data based on archive materials.*

The third study compared 50 underground mine workers exposed to diesel exhaust with 50 above-ground oil shale sector workers. It was found that miners had a higher degree of DNA damage (Comet assay data) compared to the control group (39). They had also several changes in the levels of biomarkers such as S-phenylmercapturic acid (S-PMA) and MA (28) and increased 5-aminolevulinic acid (ALA) activity (40). Muzyka et al. (40, 41) have claimed that exposure to diesel exhaust has caused changes in heme synthesis, resulting in the accumulation of ALA and protoporphyrin in miners' lymphocytes.

SELECTION OF BIOMARKERS FOR ESTONIAN HBM

Based on the analysis of pollutants, emission and monitoring, the pollutants characterizing the environmental pollution of the oil shale sector would be PM_{10}, and $PM_{2.5}$ (as well as heavy metals bound to particles), benzene and PAHs such as B(a)P.

Elvidge et al. (42) have reviewed >20 biomarkers used as $PM_{2.5}$ biomarkers. The most common of these are the markers of inflammation: C-reactive protein (CRP), interleukin 6 (IL-6), and fibrinogen. However, more than half of the studies using these markers have shown no effect. Less commonly used, but more robust biomarkers have been oxidized low-density lipoprotein (ox LDL), lipoprotein receptor-1, TBARS, which characterize lipid hyperperoxidation, and malondialdehyde (MDA), a marker of oxidative stress. However, the limitation is that these biomarkers are also elevated with cardiovascular diseases, so they are not specific for the contamination. Even more robust biomarkers are heavy metals bound to particles (43). The update of the temporal and spatial trends, should be encompassed. This should certainly include heavy metals from the earlier HBM in Estonia (lead, cadmium, mercury, copper, zinc), as well as arsenic and chromium.

In the case of benzene, we recommend to determine it as the BTEX (benzene, toluene, ethylbenzene, and xylene) metabolite complex, which has been used as a biomarker found in urine in petroleum distribution facilities (44). To assess exposure to PAH, it is valuable to use 1-OHP, which is the most commonly used PAHs biomarker in both work and living environments (45). It has been earlier used among oil shale sector workers (32).

CHEMICAL ANALYSIS OF BIOMARKERS

If the earlier studies mainly used different (AAS) techniques for heavy metal detection in blood and plasma, recently the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Inductively Coupled Plasma—Optical Emission Spectrometry (ICP-OES) have been preferred due to much lower detection limits (46). ICP-MS and ICP-OES have been applied in several studies of populations living near industrially contaminated sites (47–49).

For 1-OHP determination HPLC with fluorescence detection (50), gas chromatography with mass spectrometry (GC/MS) (51), and more recently liquid chromatography tandem mass spectrometry (LC-MS/MS) (52) have been applied using isotopically labeled standard for 1-OHP detection (53). Another important metabolite would be 1-hydroxypyrene glucuronide (1-OHP-G), which allows better determination of low exposure to PAH (54). Due to low 1-OHP-G levels, more accurate ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) should be applied (55).

For the determination of BTEX, several new studies have used headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) (56, 57). It is a sensitive methodology that can be used to determine low levels biomarkers. This is especially crucial among those less exposed, such as children (58).

DISCUSSION AND CONCLUSIONS

HBM is valuable tool in indicating exposures as well as early effects in a large number of studies (9, 12). The information on human exposure can then be linked to data on sources and epidemiological surveys in order to inform research on exposure-response relationships in humans (19).

However, there are also several limitations in the interpretation of HBM results. One of the main limitations is that in many cases it may be difficult to confirm from which source the measured effects have been induced. This can be refined by using HBM and environmental monitoring data in parallel, where we can ultimately identify the sources of the pollutants.

Other major limitations are the confounding factors that could induce similar effects, e.g., in case possible biomarkers of $PM_{2.5}$ health effects, existing cardiovascular disease might have a similar effect (42). One of the possibilities to collect data on confounding factors is through questionnaires.

Often, HBM is very costly and resource intensive. Tan et al. (59) have suggested that an HBM program could be divided into different tiers. We propose that Tier 1 could focus on exposure biomarkers as heavy metals, PAH, and BTEX metabolites. Tier 2, in a later stage, could focus on effect biomarkers, e.g., particle exposure biomarkers. Collecting blood samples in Tier 1 for Tier 2 could be beneficial.

Applying the described approached and taking into account the limitations, would warrant the solid ground for HBM in Estonia. Though, besides the exposed population, e.g., people living near oil shale industry or industry workers, the data from reference areas is needed in order to get good basis for spatial comparison. We support including the areas that have been analyzed earlier: Tallinn as capital area, Southern-Estonia as reference area and Western-Estonian coastal areas with somewhat different dietary habits like higher consumption of Baltic-Sea fish.

In the current analysis we could identify several HBM studies in the oil shale industry area in Estonia; however, all of those have been made in 1980, 1990, and 2000's. During the last two decades there has been change in environmental quality, so the update of HBM data is essential. As Estonia is recently joined HBM4EU network, this information would also be important from that perspective.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Data is available in the reports referred in the article.

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because the current study only reviews earlier studies. The original studies had been approved by local ethics committees. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HO and ML contributed conception of the study. HO and TV compiled the database of earlier studies. HO and AV contributed interpretation of the study findings. AV and KH reviewed the methodology used for chemical analysis in human biomonitoring. All authors contributed to writing the manuscript.

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Arsenic Exposure and Cancer-Related Proteins in Urine of Indigenous Bolivian Women

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Indigenous people living in the Bolivian Andes are exposed through their drinking water to inorganic arsenic, a potent carcinogen. However, the health consequences of arsenic exposure in this region are unknown. The aim of this study was to evaluate associations between arsenic exposure and changes in cancer-related proteins in indigenous women ($n = 176$) from communities around the Andean Lake Poopó, Bolivia. Arsenic exposure was assessed in whole blood (B-As) and urine (as the sum of arsenic metabolites, U-As) by inductively coupled plasma-mass spectrometry (ICP-MS). Cancer-related proteins ($N = 92$) were measured in urine using the proximity extension assay. The median B-As concentration was 2.1 (range 0.60–9.1) ng/g, and U-As concentration was 67 (12–399) μg/L. Using linear regression models adjusted for age, urinary osmolality, and urinary leukocytes, we identified associations between B-As and four putative cancer-related proteins: FASLG, SEZ6L, LYPD3, and TFPI2. Increasing B-As concentrations were associated with lower protein expression of SEZ6L, LYPD3, and TFPI2, and with higher expression of FASLG in urine (no association was statistically significant after correcting for multiple comparisons). The associations were similar across groups with different arsenic metabolism efficiency, a susceptibility factor for arsenic toxicity. In conclusion, arsenic exposure in this region was associated with changes in the expression of some cancer-related proteins in urine. Future research is warranted to understand if these proteins could serve as valid biomarkers for arsenic-related toxicity.

Keywords: arsenic, Andes, Bolivia, FASLG, TFPI2, biomarker, carcinogenic

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INTRODUCTION

Inorganic arsenic (iAs) is classified as a class I human carcinogen (1). Chronic exposure to iAs has been associated with multiple types of cancer, including cancers of the skin, lung, bladder, kidney, liver, and prostate (1–4). Exposure to iAs has also been associated with non-carcinogenic health effects such as cardiovascular disease, diabetes, and immunotoxicity (4–6).

Leakage of iAs from volcanic bedrocks into groundwater used as drinking water is one of the main contributors to human exposure, especially in Latin America where more than 14 countries present elevated iAs in drinking water (7). Arsenic-related cancer has been evaluated in some Latin American countries, mainly Chile, Argentina, and Mexico (6). In the Chilean city of Antofagasta

and in the Córdoba province of Argentina, studies have consistently found an increased lung, bladder, and kidney cancer mortality in relation to chronic exposure to iAs (8–10). Recently, iAs was linked to laryngeal cancer mortality in Chile (11), and chronic exposure to iAs and risk for breast cancer was evaluated in Latin America (12–14). In the Bolivian Andes, we have shown that women living around Lake Poopó are exposed to iAs (15). However, very little is known about cancer or any toxicity in relation to iAs exposure in Bolivia.

A growing body of knowledge supports the use of urinary proteins to detect early signs of cancer and other diseases (16–19). Although several epidemiological studies have investigated protein biomarkers associated with iAs in humans (20–22), the relation between urinary proteins and arsenic remains unexplored. To our knowledge, only one study has previously explored urinary proteins in relation to iAs exposure. In that study, limited to proteins within 2–10 kDa of size, highly exposed individuals (>100 μg/L U-As) from the United States and Chile had decreased expression of human beta-defensin 1 (HBD-1), an antimicrobial protein and potential tumor suppressor gene in urological cancers (23). The effect of iAs on urinary proteins has been overlooked in the scientific literature despite its potential for biomarker discovery. Therefore, the aim of this cross-sectional study was to evaluate the associations between putative cancer-related proteins in urine and arsenic exposure in indigenous individuals from the Bolivian Andes.

MATERIALS AND METHODS

Study Participants and Sample Collection

We recruited women living in villages around Lake Poopó, located in the southern region of the Bolivian highlands, at 3,686 m above sea level (15). This area is characterized by arsenic-rich soils, common in the Andean regions. Recruitment of 201 women took place during five field trips organized between September 2015 and November 2017 from a total of 10 villages around Lake Poopó. Women with no major health conditions were recruited on a voluntary basis (one woman with a brain tumor and tuberculosis was excluded). We recruited women from two ethnic groups, Aymara-Quechua and Uru, that inhabit this region. During recruitment, the women had similar eating habits and lifestyles in all villages, based on questionnaire data. Men were not recruited into the study because they usually worked away from their village leading to a different arsenic exposure. For this study, we started from a subset of women ($n = 182$) for which we had most complete biological data, including genetic data which is not presented in this study. Out of these women, we excluded five that presented extreme levels of nitrites in urine (based on urine strip determination described further on), and one woman without urine strip data. In total, 176 women were included in the current study, of which 149 identified themselves as Aymara-Quechua and 27 as Uru. This study was approved by the Comité Nacional de Bioética (Bolivia) and the Regional Ethic Committee of Karolinska Institutet (Sweden). Prior to recruitment and sampling, participants were informed orally and in written form about the project, and signed informed consent was obtained.

We carried out personal interviews including information such as age, ethnicity, weight, height, blood pressure (measured in sitting position), health status, and frequency of chewing coca leaves (15). Body mass index was not used since Andean populations are known to have higher values as a consequence of living at high altitude without them being associated with excess body fat (15, 24, 25). Spot urine samples were collected throughout the day during the field trips. The women were given instructions on how to collect urine samples, including wet wipe cleaning and mid-stream urine collection to minimize contamination. The urine samples were collected in 20 mL polyethylene bottles, previously confirmed as free of trace elements. Venous blood samples were obtained with BD Vacutainer Eclipse blood collection needles (Becton Dickinson, USA) in Trace Elements NH Sodium Heparin tubes (Vacuette, Greiner Bio, Austria), or in Lithium Heparin tubes (Vacuette) when the Sodium Heparin tubes were not available. Due to field trip limitations, it was not possible to separate plasma. Urine and blood samples were stored directly after sampling at −18°C in a portable freezer (ARB, Australia), and stored at Universidad Mayor de San Andrés (La Paz, Bolivia) at −20°C until further shipment. Samples were transported on dry ice to Karolinska Institutet (Stockholm, Sweden), where they were stored at −20°C for long-term storage. All urine and blood samples had similar freeze-thaw cycles.

Protein Measurements in Urine

Urine samples for protein measurements were thawed overnight at 4°C. After thoroughly mixing each sample, it was aliquoted, debris were separated by centrifugation (845 g for 10 min), and 50 μL of the supernatant was added to 96-well plates (Thermo Fisher Scientific, USA) covered with MicroAmp Clear Adhesive Film (Thermo Fisher Scientific). Samples were transported on ice from Karolinska Institutet (Stockholm, Sweden) to Olink Proteomics (Uppsala, Sweden).

Urine samples were analyzed with the Proseek Multiplex Oncology II panel ($N = 92$ proteins) based on the Proximity Extension Assay (PEA) developed and performed by Olink Proteomics. The PEA is a dual-recognition immunoassay which uses a pair of antibodies labeled with DNA oligonucleotides specific for each pre-determined protein on the panel. When both labeled antibodies bind to the target protein, the oligonucleotide labels are in enough proximity to hybridize and create a PCR target sequence that will be quantified by real-time PCR. The Oncology II panel includes proteins belonging to biological process ontologies relevant to cancer development: angiogenesis ($n = 20$), apoptotic process ($n = 34$), cell adhesion ($n = 35$), cell differentiation ($n = 42$), cell motility ($n = 30$), cell proliferation ($n = 43$), cellular metabolic process ($n = 47$), cellular response to stress ($n = 23$), chemotaxis ($n = 14$), extracellular matrix organization ($n = 9$), immune response ($n = 27$), MAPK cascade ($n = 25$), proteolysis ($n = 19$), response to hypoxia ($n = 3$), and other gene ontology terms ($n = 9$). Methodological details, data processing, quality control and normalization are described by Assarsson et al. (26) and are available online at https://www.olink.com. Protein level data obtained by the Olink Proteomic facility are presented as Normalized Protein eXpression (NPX)

values, which have arbitrary units on a log2-scale. The intra-assay coefficient of variance (CV%) was below 20% for all proteins (only one protein above 15%), while the inter-assay CV% was below 30% for all proteins (only two proteins between 20 and 30%). The limit of detection (LOD) for each protein assay is defined as three times the standard deviation above background level based on internal controls. For downstream analyses, we included proteins that had more than 40% of the samples above the LOD, i.e., 45 proteins. Since this multiplex assay was initially developed to detect proteins in plasma, we expected that some of the selected proteins in the panel would not be present to the same extent in urine. Therefore, we were less stringent with the LOD cut-off compared to other studies that included proteins measured in plasma with more than 80–90% of the samples above LOD (27, 28).

Arsenic Exposure and Metabolism Efficiency Assessment

For this study, we used arsenic concentrations in whole blood (B-As), and not in urine, as a biomarker of exposure in order to avoid the potential co-excretion of arsenic and proteins in urine (29).

We previously described that the women in this region had elevated concentrations of arsenic and lithium in urine, and that arsenic and lithium concentrations in urine were correlated ($r_S = 0.47$, p-value < 0.001) (15). Therefore, in this study we also used lithium concentrations in whole blood (B-Li; correlation with B-As: $r_S = 0.44$, p-value < 0.001) to evaluate potential confounding.

The blood samples were prepared for inductively coupled plasma-mass spectrometry (ICP-MS; operating conditions in **Supplementary Table 1**) by a direct alkali dilution method (30). Briefly, blood samples were diluted 1:17–44 with an alkali solution consisting of 2% butanol (Honeywell Research Chemicals, Germany), 0.05% EDTA (Sigma-Aldrich, USA), 0.05% Triton X-100 (Sigma-Aldrich), 1% NH_4OH (Romil, UK), and 20 μg/g internal standards ^{45}Sc, ^{72}Ge, ^{103}Rh, ^{175}Lu, and ^{193}Ir. Before analysis, the diluted samples were sonicated for 5 min, and centrifuged at 694 g for 5 min. The Agilent 7900 ICP-MS (Agilent Technologies, Japan) equipped with an octopole reaction system (ORS) collision/reaction cell technology was used for measuring concentrations of arsenic and lithium. The LOD for each element was determined as three times the standard deviation of analyzed blanks (alkali solution) and as signal/noise = 3. The limit of quantification (LOQ) was determined as 10 times the standard deviation of analyzed blanks. The analysis precision was estimated by measuring two in-house blood samples in triplicate. As quality control, two commercially available whole blood reference materials were analyzed: Seronorm™ Trace Elements Whole Blood L-1 (LOT 1702821) and L-2 (LOT 1702825), and the obtained average arsenic values (1.9 ± 0.1 μg/kg and 10 ± 1 μg/kg) were in agreement with the reference values (2 ± 0.4 μg/kg and 11.6 ± 2.4 μg/kg). Certified values were converted from μg/L to μg/kg by dividing by the average density of blood (1.055 kg/L). In addition, the reference materials were spiked with 2–1385 μg/kg lithium, and on average 101% of the added lithium was recovered. Blanks and reference materials were treated together with the collected whole blood samples and analyzed in the beginning, in the middle, and at the end of each analysis. For some individuals, blood samples for element analysis were collected in Lithium Heparin tubes instead of Trace Elements NH Sodium Heparin tubes (Vacuette, Greiner Bio), and therefore B-Li results could not be obtained for all individuals. Leach tests for both types of Vacuette tubes were performed, and no traces of arsenic were detected in neither of them (data not shown).

In the human body, iAs is metabolized via the one-carbon cycle by reducing As(V) to As(III), and methylating As(III) into methylarsonic acid (MMA) and further into dimethylarsinic acid (DMA). The metabolism is not complete, and all four arsenic species are to varying degrees excreted in urine (31). We previously measured arsenic in urine, both as total arsenic in urine (including organic forms such as arsenobetaine from seafood) and as the sum of iAs metabolite concentrations (iAs + MMA + DMA; U-As) in urine, and concluded that this study group is mainly exposed to inorganic forms (15).

Covariates

Urine test strips (Combur-7 Test strips, Roche, Switzerland) were used immediately after sample collection to determine urinary pH, glucose, ketones, leukocytes, nitrites, proteins, erythrocytes, and hemoglobin. These urine reagent strips are commonly used in clinical settings to detect diabetes and other kidney and urinary tract diseases in a semiquantitative manner. Results were obtained and graded on a discrete numerical scale (0, 1, 2, 3, or 4) by comparing the color of the patches on the strip with the colors on the label, according to the manufacturers indications.

To account for variations in urine dilution, we previously measured specific gravity and osmolality (15). In this study, we only included osmolality since it is strongly correlated with specific gravity in the study group ($r_S = 0.99$, p-value < 0.001), and because specific gravity can be influenced by the presence of proteins and glucose (32).

We evaluated if differences in storage time affected the relative protein concentrations by including the field trip as a covariate (∼26 months between the first and the last sampling occasion).

We also assessed if the association between B-As and cancer-related proteins was influenced by the efficiency of arsenic metabolism, determined as the relative concentration (%) of the different iAs metabolites in urine. Higher fractions of iAs and MMA in urine are associated with higher risk of adverse health outcomes, and therefore a low efficiency of arsenic methylation is considered a susceptibility factor for arsenic toxicity (2, 33). We stratified the study group as below and equal, or above median %MMA.

Statistical Analyses

All analyses were performed with RStudio (version 1.1.423) using R (version 3.6.2). General characteristics of the study group that are continuous variables and protein levels as NPX values are presented as median and interquartile range (IQR), while categorical variables are presented as percentage or number of individuals per category. NPX values below LOD were excluded from the dataset.

Linear models of principal components were analyzed to assess the influence of different covariates on the overall protein variation. For this, we used the *prince* and *prince.plot* functions within the *swamp* package. These functions perform a principal component analysis for the overall protein expression variation and then perform a linear regression between each variable and principal component to evaluate their association. The heat map presents -$\log_{10}$(*p*-value) for the associations, and variables were hierarchically clustered using the *hclust* function. Since the *prince* function does not handle missing data, and imputation was not optimal due to limited input data, we used the NPX dataset substituting values below the LOD by the LOD value specific for each protein. Spearman correlation-tests were carried out between the covariates that were statistically significant in the linear model of principal component analyses. Covariates that were significantly associated with at least one of the 10 principal components and not correlated between each other, as assessed by Spearman correlation-test, were further included in linear regression models used to evaluate individual protein variation and to assess the relation between these proteins and B-As (see below).

To evaluate how much the selected covariates explained the variation of each protein, univariate linear regression analyses were performed. Each model included one protein (as dependent variable) and one covariate (as independent variable) at a time. The variance explained (R^2) was presented as stacked bar plots. To assess the association between B-As and protein expression, multivariable-adjusted linear analyses were performed, adjusting for the covariates that were significantly associated with the overall protein variation. Standardized beta coefficients were obtained for all covariates in the model and presented in stacked bar plots. One individual was excluded from these analyses since no B-As data was available. Similar analyses were performed also adjusting for B-Li as sensitivity analyses. The top associated proteins with B-As were further evaluated with Spearman pairwise correlations, with scatter plots against B-As including a stratification by arsenic metabolism efficiency, and with boxplots to compare between low and high exposed participants. The categorization between low and high exposed was done by splitting by the median B-As. Since the urinary proteins were not normally distributed, we performed Wilcoxon-tests to evaluate the differences between groups.

RESULTS

Characteristics of the Study Participants

Information on the women's anthropometric and lifestyle characteristics, urinary characteristics, and exposure biomarkers are included in **Table 1**. The exposure to iAs from drinking water of the current study group (U-As median 67 μg/L, range 12–399 μg/L; B-As median 2.1 ng/g, range 0.60–9.1 ng/g) is comparable to that of the whole study population ($N = 201$ women, U-As median 65 μg/L, range 12–407 μg/L) (15). Furthermore, U-As (as sum of metabolites, adjusted for average osmolality) and B-As were strongly correlated ($r_S = 0.85$, *p*-value < 0.001; **Supplementary Figure 1**) reflecting that both matrices are reliable biomarkers for iAs exposure in this study group. Only two women reported consuming alcoholic beverages frequently (every other week), and seven reported smoking tobacco (yes/no). Based on the urine strips, no woman had ketones, two had elevated glucose, 10 nitrites, 16 proteins, 40 hemoglobin, and 12 erythrocytes in urine.

TABLE 1 | Characteristics of the individuals included in the study ($n = 176$).

	n	Median (IQR)[a]
General characteristics		
Age (years)	176	36 (28–48)
Weight (kg)	176	60 (53–70)
Height (cm)	176	149 (146–153)
Coca chewing (yes)[b]	174	74%
Systolic blood pressure (mmHg)	175	100 (90–110)
Diastolic blood pressure (mmHg)	175	70 (60–70)
Urine characteristics		
Specific gravity	176	1.02 (1.01–1.02)
Osmolality (mOsm/kg)	176	764 (564–883)
Urine pH	176	5.5 (5.0–6.1)
Urine leukocytes (0/1/2/3)[c]	176	146/10/17/3
Exposures		
U-As (μg/L)[d]	176	67 (47–111)
iAs (%)	176	12 (8.6–17)
MMA (%)	176	7.7 (6.2–9.6)
DMA (%)	176	79 (74–84)
B-As (ng/g)[e]	175	2.1 (1.4–3.0)
B-Li (ng/g)[e]	159	18 (9.4–30)

[a] *Data presented as median and interquartile range (IQR) unless otherwise stated.*
[b] *Percentage of individuals who reported chewing coca leaves regularly.*
[c] *Number of individuals in each category of urine leukocyte levels, classified according to the urine strips Combur 7 Test.*
[d] *Sum of urinary iAs metabolites adjusted for average urinary osmolality (727 mOsm/kg) of the total study population as described in De Loma et al. (15).*
[e] *Measured in whole blood.*

Covariates Explaining Protein Variation in Urine

Descriptive statistics of the 92 cancer-related proteins measured by the Multiplex Oncology II panel are shown in **Supplementary Table 2**. Out of the proteins initially measured, 45 proteins were further evaluated, i.e. those with $>$ 40% of observations above LOD. To assess to which extent characteristics of the study participants were associated with variation of these proteins, we performed linear regression models of principal components (**Supplementary Figure 2**). Age, urinary osmolality, urinary leukocytes and urinary pH significantly explained the overall protein variation in urine. Urinary pH and osmolality were correlated ($r_S = -0.21$, *p*-value $= 0.005$), while none of the other covariates presented pairwise correlations, and therefore urinary pH was not included in further analyses. No significant associations were found for coca chewing, ethnicity, or sampling field trip. None of the other markers from the urine strips (glucose, nitrites, proteins, hemoglobin, nor erythrocytes) were associated with the overall protein variation of the cancer-related proteins. In addition, there

were no differences in protein expression between ethnicities (data not shown).

To evaluate the protein variance explained by each covariate independently, we performed linear regression analyses including each covariate individually (age, urinary osmolality, urinary leukocytes, and B-As as the exposure of interest; **Figure 1A**). Urinary osmolality explained the variation of most proteins measured in urine, in some cases explaining up to 49%

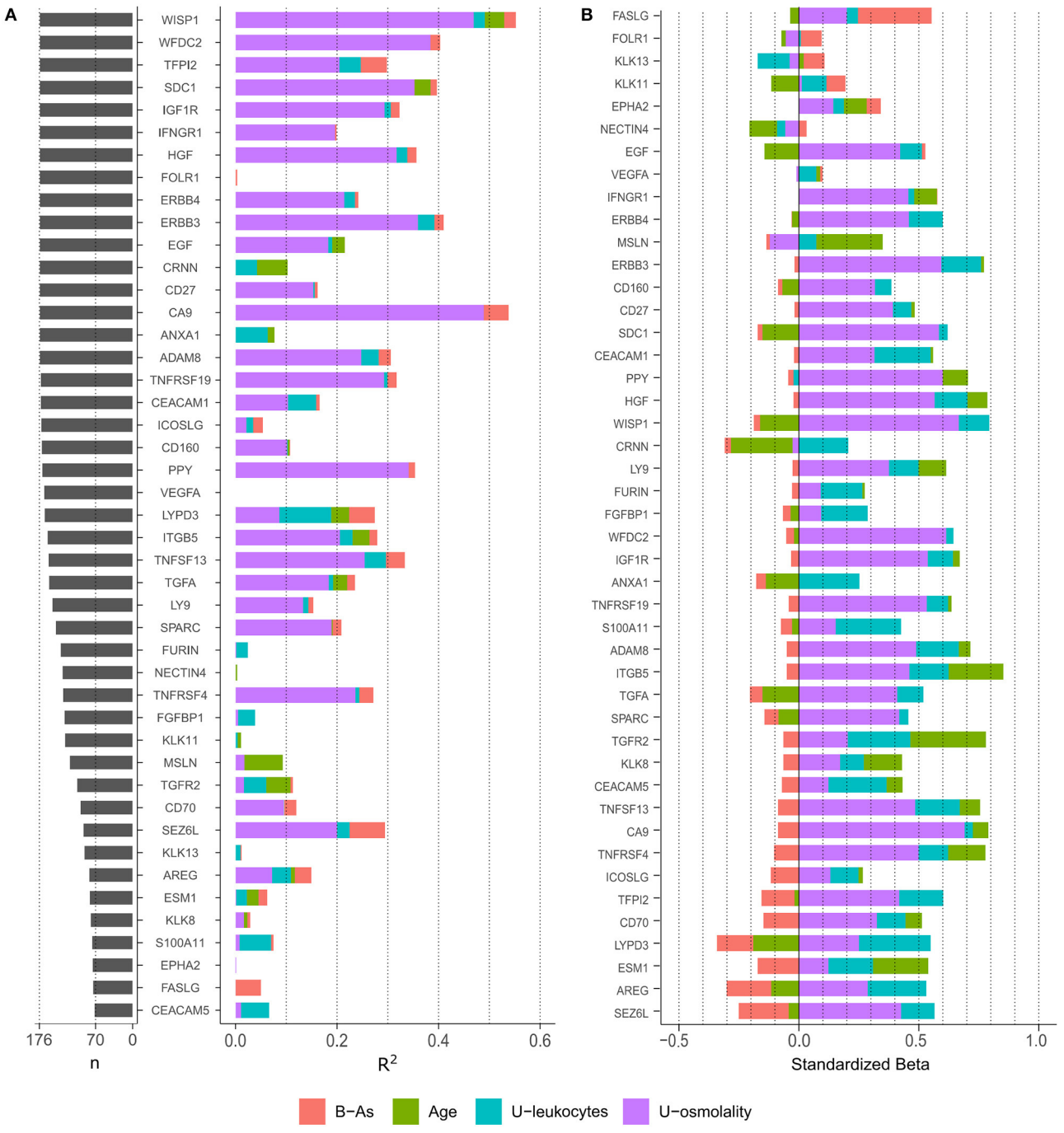

FIGURE 1 | (A) Number of observations (*n*) for each protein and variance explain (R^2) by each variable in independent linear regression models, including one independent variable in the model at a time. Only proteins with > 40% of observations above LOD were included. **(B)** Standardized effect of each variable on protein levels. The plot depicts the standardized beta coefficient of each variable (as independent variables) in the multivariable-adjusted linear regression model including the protein as dependent variable. Proteins in the plot are ordered by the effect of B-As on protein levels (B coefficient for B-As).

of the variation. However, 13 proteins were not influenced by osmolality (R^2 < 1%: FOLR1, CRNN, ANXA1, VEGFA, FURIN, NECTIN4, FGFBP1, KLK11, KLK13, ESM1, S100A11, EPHA2, and FASLG). Age explained the variation (R^2: 1.3–7.5%) of 11 proteins: WISP1, SCD1, EGF, CRNN, ANXA1, LYPD3, ITGB5, TGFA, MSLN, TGFR2, and ESM1. The only marker selected from the urine strip test was urinary leukocytes, as a proxy for inflammation of the urinary tract, which explained between 1 and 10% of the variation of 23 proteins of which LYPD3, ANXA1, and S100A11 showed the highest R^2 (10, 6.3, and 6.2% respectively). B-As explained 5–7% of the protein variation of TFPI2, LYPD3, SEZ6L, and FASLG.

Urinary Proteins Associated With Arsenic Exposure

We further examined how B-As was associated to cancer-related proteins in urine by multivariable linear regression models adjusted for age, urinary leukocytes, and urinary osmolality. Out of the 45 proteins, four were associated (p-value < 0.05) with B-As: Tumor necrosis factor ligand superfamily member 6, FASLG; Seizure 6-like protein, SEZ6L; Ly6/PLAUR domain-containing protein 3, LYPD3; and Tissue factor pathway inhibitor 2, TFPI2 (**Figure 1B**, **Table 2**, and **Supplementary Table 3**). None of the associations were statistically significant after adjusting for multiple testing. The relative protein expression of SEZ6L, LYPD3, and TFPI2 decreased with higher B-As concentrations, while FASLG increased with increasing B-As concentrations (**Table 2** and **Figure 2A**). Differences in arsenic metabolism efficiency did not change the association between these top proteins and B-As (**Figure 2B**). In addition, we explored if these top proteins were significantly different between individuals with low or high arsenic exposure, by splitting by median B-As (**Figure 2C**). Individuals highly exposed to arsenic had significantly lower SEZ6L, LYPD3, and TFPI2 levels (p-value = 0.021, 0.0001, 0.004, respectively). Highly exposed individuals had higher levels of FASLG, although not significantly (p-value = 0.09).

We also explored the correlation between the top proteins that were associated with B-As. Spearman pairwise correlations between SEZ6L, LYPD3, and TFPI2 were observed, but not for FASLG (**Supplementary Figure 3**). We considered performing downstream analyses, e.g., pathway enrichment, but the number of proteins associated with B-As was not enough for such analyses to be suitable. Sensitivity analyses were performed including B-Li in the models. The effect estimates for the associations between B-As and FASLG, SEZ6L, LYPD3, and TFPI2 did not change more than 10%, although they were no longer significant (p-value < 0.05), probably due to the decrease in sample size (**Table 2** and **Supplementary Table 3**).

TABLE 2 | Top 10 proteins in urine associated with B-As.

	Main analysis[a]			
Protein	***n***	**R^2 (%)**	**B (95% CI)**	***p*-value**
FASLG	74	5	0.155 (0.034, 0.276)	0.013
SEZ6L	93	25	−0.038 (−0.072, −0.004)	0.028
LYPD3	166	23	−0.172 (−0.331, −0.014)	0.034
TFPI2	175	24	−0.125 (−0.249, −0.001)	0.049
AREG	82	15	−0.096 (−0.204, 0.012)	0.080
CA9	175	49	−0.05 (−0.112, 0.013)	0.119
ESM1	81	7	−0.067 (−0.152, 0.019)	0.124
ICOSLG	172	3	−0.097 (−0.225, 0.03)	0.134
CD70	98	11	−0.046 (−0.106, 0.015)	0.137
TNFRSF4	131	27	−0.066 (−0.164, 0.032)	0.183

Data presented as B coefficients and 95% confidence intervals (CI) for the association with B-As.

No proteins passed the Bonferroni adjustment for multiple comparisons (p-value threshold = 0.05/45 = 0.001).

[a]*Multivariate linear regression models adjusted for age, urinary osmolality, and urinary leukocytes.*

DISCUSSION

To our knowledge, this study is the first to evaluate the toxicity of environmental iAs exposure in individuals from Bolivia. We identified four proteins (FASLG, SEZ6L, LYPD3, and TFPI2) measured in urine that were associated with B-As in indigenous women from the Bolivian Andes. Arsenic concentrations in blood were positively associated with relative protein expression of FASLG and inversely associated with SEZ6L, LYPD3, and TFPI2. These associations were not statistically significant when correcting for multiple comparisons likely due to limited sample size. When comparing individuals with high vs. low exposure to arsenic, SEZ6L, LYPD3, and TFPI2 were significantly decreased in the high exposure group, while FASLG was non-significantly increased. The individual proteins are discussed below including previous relations reported for arsenic, when known. So far, there is no knowledge about whether urinary levels of these proteins are predictive of cancer. Longitudinal research is needed to establish whether these proteins serve as early biomarkers of arsenic-related carcinogenicity, or if these associations are a consequence of an adaptive response to the exposure. This study also identified other influential factors for the variation in protein expression in urine, such as urinary dilution and age, which is important information for future use of these proteins as potential risk biomarkers.

FASLG binds to Fas receptors and regulates an apoptotic signaling pathway (34, 35). FASLG has a dual function; it both promotes and inhibits cell death depending on the cell type (36). Elevated serum concentrations of soluble FASLG have been detected in patients with leukemia, lymphoma and multiple solid tumors (37). Arsenic has been linked to FASLG protein expression, mainly in *in vitro* studies of the cancer drug arsenic trioxide, which is currently used for treatment of acute promyelocytic leukemia. Different leukemia cell lines exposed to arsenic trioxide showed increased gene or protein expression of FASLG (38–40). Human studies of environmental exposure to iAs and FASLG are few and inconclusive. Women highly exposed to iAs (median U-As 276 μg/L) from the Argentinean Andes presented higher *FASLG* gene expression in sorted T-cells compared to women with lower exposure (median U-As 65 μg/L)

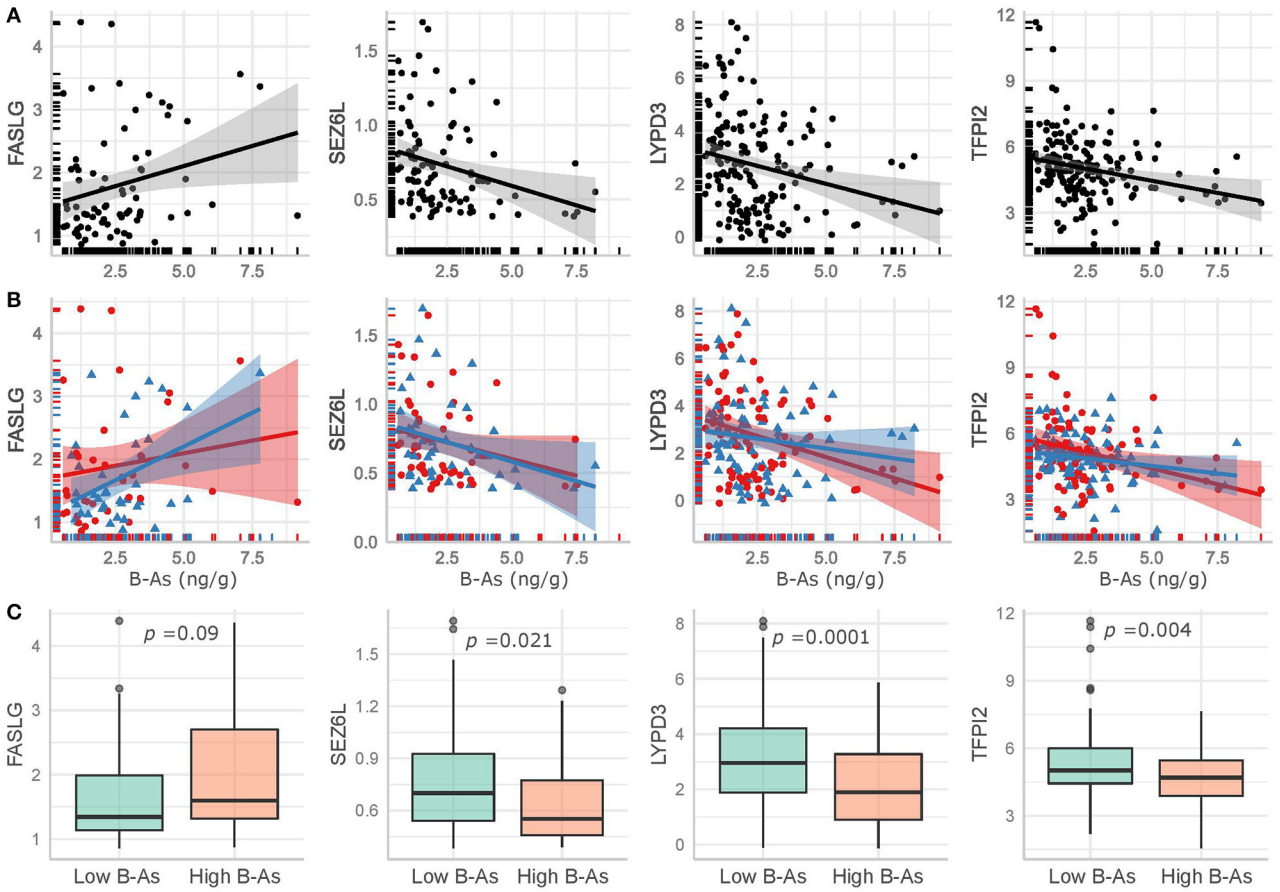

FIGURE 2 | Top proteins in urine significantly associated (p-value < 0.05) with B-As in the adjusted linear regression models, including non-adjusted linear regression estimates and their confidence intervals for **(A)** the total study group and **(B)** stratified by arsenic methylation efficiency, as below (red circles and line) or above (blue triangles and line) the median %MMA. **(C)** Comparison of the top proteins in urine between low and high exposed individuals, divided by the median B-As. The p-values for Wilcoxon-tests are included.

(41). In contrast, five individuals from Mexico exposed to iAs (median 224.3 mg/g creatinine) showed lower gene expression of *FASLG* in peripheral blood mononuclear cells (including T cells, B cells, and NK cells) compared to five unexposed individuals (iAs < 2.2 mg/g creatinine) (42).

TFPI2 is an inhibitor of the tissue factor pathway involved in blood coagulation. Gene expression of *TFPI2* was decreased in samples of bladder tumors (43) and cervical tumors (44), while TFPI2 protein concentrations in serum were higher in ovarian clear cell adenocarcinoma patients (45, 46). Hypermethylation, generally associated with decreased gene expression (47), was detected for *TFPI2* in multiple types of cancer compared to normal adjacent tissues (48–52). A human prostate epithelial cell line transformed by chronic exposure to arsenite presented decreased *TFPI2* gene expression (53), in line with our findings. On the contrary, normal human lung cells exposed to sodium arsenite for 30–60 days showed an upregulation of *TFPI2* gene expression (54).

Two of the proteins identified, SEZ6L and LYPD3, have to our knowledge not been related to arsenic before. SEZ6L is a transmembrane protein in the endoplasmic reticulum and the cell membrane whose function is still unclear. In our study, lower urinary expression of SEZ6L was associated with increasing arsenic exposure. Interestingly, deletions in the *SEZ6L* gene have been found in primary lung tumor cells (55), and loss-of-function variants in *SEZ6L* have been associated with increased risk of lung cancer (56). *SEZ6L* is also reported as a fusion gene in some lung cancers, melanoma and skin cancer (57).

LYPD3 is a glycosylphosphatidylinositol-anchored urokinase receptor involved in cell-matrix interactions and metastasis (58, 59). In contrast to our findings of lower relative protein expression in relation arsenic exposure, enhanced protein expression of LYPD3 has been identified in tissues from urothelial cancers (59), breast cancers (60), melanoma (61), and lung cancers (62). Recently, high protein expression levels of LYPD3 in tumor tissues have been recognized as a biomarker of poor prognosis for lung cancer patients (63, 64).

We also identified factors that explained the overall variation of the 45 proteins measured in urine, such as age, urinary osmolality, and urinary leukocytes. This highlights the importance to take these factors into account when evaluating protein biomarkers in urine. Since urinary pH and osmolality

were correlated to some extent in this study group, we did not adjust the final models for urinary pH. A study with Sprague-Dawley rats did not show any effect of urinary pH on urinary proteins determined with urine test strips either (65). Although urinary osmolality is less affected by proteins in urine than other dilution markers such as specific gravity (66), it is not possible to discern the direction of the association between the proteins in this study and osmolality. Storage time of the samples may affect some proteins in plasma (67). In the current study, storage time did not explain the overall protein variation or influenced the top proteins associated with B-As. There were several women with menstrual bleeding, but this did not influence the proteins evaluated. Women with urinary tract infection, identified by the presence of leukocytes in urine, were expected considering the limited access to health care in the study area. However, this did not influence the variation of the proteins.

Regarding the choice of matrices for measuring exposure to arsenic, B-As is most suitable to assess recent exposure to iAs since it is rapidly cleared from this matrix (68). Still, the strong correlation between B-As and U-As concentrations in the current study group likely reflects a chronic exposure to iAs, and it allowed us to use B-As as a valid biomarker of exposure. Arsenic concentrations in drinking water from these villages did not present temporal variations during the 2 years of recruitment, supporting that these women were constantly exposed to iAs (15). By using two different biological matrices to measure exposure (blood) and effect biomarkers (urine), we avoided potential problems of co-excretion in urine, previously identified for other elements like cadmium (29). Furthermore, it would be valuable to analyze these cancer-related proteins in other matrices such as serum or plasma in order to correlate these with urine values, since no data on this is available. Unfortunately, the sampling of plasma from the study individuals was not optimal for protein analysis.

The detection of proteins in urine is commonly considered an indication of kidney disease. High levels of proteins in urine, also known as proteinuria, have been associated with exposure to arsenic (69). In the review by Zheng et al. (69), all studies evaluating proteinuria used U-As as a biomarker of exposure, therefore not being exempt from the potential problem of co-excretion between biomarkers of effect and exposure in the same matrix. The constant development of more advanced protein detection techniques in urine demonstrates that urine is more protein-rich than previously believed, even under normal conditions (70). This, and the fact that urine is an abundant and non-invasive sampling matrix, justifies the attempts to identify more disease biomarkers in urine. In fact, urinary protein biomarkers have been identified as early diagnostic markers for several cancer types (16–18, 71). Regarding the Proximity Extension Assay used in this study, only one other study has employed this technology to evaluate protein expression in urine (72). In Fellström et al. (72), they found an association between lipid markers in serum and inflammation- and cardiovascular-related urinary proteins in 75-year-old individuals, but urinary dilution was not adjusted for in the analyses.

This study group in the Bolivian Andes has a markedly efficient arsenic metabolism capacity (15), which may influence the degree of arsenic toxicity. This, and the fact that cancer types differ between populations depending on their underlying genetic background and susceptibility (73), highlights the need for population-specific studies about arsenic toxicity. This work is a cross-sectional study of apparently healthy women, and therefore we cannot distinguish if the observed variations are adaptive or toxic responses to arsenic exposure. In addition, since iAs is also nephrotoxic (69), it is not possible to disentangle if the associations found are due to arsenic-induced renal toxicity altering protein excretion, or if these proteins serve as proxy for arsenic toxicity in other organs. More research is warranted about the relation between these proteins in different matrices and arsenic-related toxic outcomes for these proteins to be used as toxicity biomarkers or to elucidate the toxicity mechanisms of arsenic. The strengths of this study are the well-characterized arsenic exposure and the use of multiplex proteomic technologies to identify novel candidates to study arsenic-related health effects.

CONCLUSION

Using multiplex proteomic methods in urine samples, we identified four putative cancer-related proteins (FASLG, SEZ6L, LYPD3, and TFPI2) associated with arsenic exposure in women living around Lake Poopó, Bolivia. In order to clarify if these proteins represent early arsenic-related carcinogenic changes, follow-up studies are needed. By exploring cancer-related proteins in urine, we hope to contribute to the future development of disease and/or toxicity biomarkers with non-invasive sampling methods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Nacional de Bioética (Bolivia) and the Regional Ethic Committee of Karolinska Institutet (Sweden). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG, NT, and KB planned and designed the research. JD, FA, JG, NT, and KB recruited the study participants and collected the data. ML performed the metal analysis. JD assisted by AG conducted the statistical analyses. JD and KB wrote the manuscript. All authors proofread and commented on previous versions of the manuscript and approved the final version of it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2020.605123/full#supplementary-material

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A Novel Transcriptome Integrated Network Approach Identifies the Key Driver lncRNA Involved in Cell Cycle With Chromium (VI)-Treated BEAS-2B Cells

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Hexavalent chromium [Cr(VI)] is a well-known occupational carcinogen, but the mechanisms contributing to DNA damage and cell cycle alternation have not been fully characterized. To study the dose-response effects of Cr(VI) on transcription, we exposed BEAS-2B cells to Cr(VI) at concentrations of 0.2, 0.6, and 1.8 μmol/L for 24 h. Here, we identified 1,484 differentially expressed genes (DEGs) in our transcript profiling data, with the majority of differentially expressed transcripts being downregulated. Our results also showed that these DEGs were enriched in pathways associated with the cell cycle, including DNA replication, chromatin assembly, and DNA repair. Using the differential expressed genes related to cell cycle, a weighted gene co-expression network was constructed and a key mRNA-lncRNA regulation module was identified under a scale-free network with topological properties. Additionally, key driver analysis (KDA) was applied to the mRNA-lncRNA regulation module to identify the driver genes. The KDA revealed that ARD3 (FDR = 1.46×10^{-22}), SND1 (FDR = 5.24×10^{-8}), and lnc-DHX32-2:1 (FDR = 1.43×10^{-17}) were particularly highlighted in the category of G2/M, G1/S, and M phases. Moreover, several genes we identified exhibited great connectivity in our causal gene network with every key driver gene, including CDK14, POLA1, lnc-NCS1-2:1, and lnc-FOXK1-4:1 (all FDR < 0.05 in those phases). Together, these results obtained using mathematical approaches and bioinformatics algorithmics might provide potential new mechanisms involved in the cytotoxicity induced by Cr.

Keywords: hexavalent chromium, transcriptome, cell cycle, lncRNA, regulation network

INTRODUCTION

Inhalation exposure to hexavalent chromium [Cr(VI)] has been recognized as a significant occupational carcinogen according to the final document from the National Institute for Occupational Safety and Health (NIOSH) (NOISH, 2008). Several researchers have explored the underlying molecular mechanisms induced by Cr(VI) related to cellular transformation (Karaulov et al., 2019) and tumorigenesis (Holmes et al., 2008; Wang et al., 2019), whereas the transcriptomic responses remain elusive. Of all the possible mechanisms of carcinogenesis associated with exposure to Cr(VI), it is hard to ignore the effects of epigenetic modifications and cytogenetic damage (Rager et al., 2019), mainly the regulatory signaling pathways related to the two processes described above.

To better understand the history of transcriptome studies in the field, a brief bibliometric study was conducted to identify trends based on the frequently occurring keywords in published papers from different years. This study clearly demonstrated the changes in the main topics in this area during the last half-century. Since the first report of cancer among a population of Cr-exposed workers, Cr(VI) compounds have remained on the list of potential threats to human health. Before 1995, several studies laid a profound foundation in the toxicity field, especially regarding DNA expression under the condition of exposure to multiple metals (i.e., with nickel) (Alcedo et al., 1994) and the proposed "uptake-reduction" model, which suggests the hypothesis that molecular events of genes may induce gene expression changes in carcinogenicity (Wetterhahn and Hamilton, 1989; Dubrovskaya and Wetterhahn, 1998) and DNA damage (Standeven and Wetterhahn, 1989). From 1995 to 2000, researchers mainly focused on the toxicology of lung cells, including type II pneumocytes (Shumilla and Barchowsky, 1999) and fibroblasts (Carlisle et al., 2000), and the mechanisms of apoptosis induced by genetically programmed cell death or the effect of transcriptional inhibition (Singh et al., 1998; Röpke et al., 2000). From 2000 to 2005, Cr-induced apoptosis became the most debatable topic. Researchers studied the possible sensors or mediators involved in apoptosis, particularly the effect of ATM protein and p38 MAP kinase (Ha et al., 2003; Wakeman et al., 2005). In addition, the role of free radicals following Cr(VI)-induced DNA damage and carcinogenesis was speculated (Liu et al., 2001; Zhang et al., 2001). From 2006 to 2010, the potential mechanisms of Cr(VI) carcinogenesis in lung cells were extensively published, and the main hypothesis was related to genomic instability (Holmes et al., 2008), including microsatellite instability (Hirose et al., 2002; Takahashi et al., 2005), numerical chromosome instability (Xie et al., 2005), and consequences of the imbalance between cellular damage and repair systems (Yao et al., 2008). Then, by the year 2010, the Environmental Working Group had detected Cr-polluted drinking water in 42 states that affected 74 million Americans (Sutton, 2010), resulting in calling for a legal limit for Cr(VI) and studies investigating the environmental carcinogenicity targeted toward the digestive system (Stern, 2010; Kopec et al., 2012; Thompson et al., 2014). Additionally, the successful discovery of microRNAs (miRNA) (Hobert, 2008) provided a new perspective for gene regulation research. As a result, miRNA studies related to Cr were introduced into the field of environmental toxicology, and they aimed to elucidate the mechanisms of lung cancer induced by Cr (He et al., 2013; Li et al., 2014). Over the last 4 years, miRNA-related research has been a topic of high interest and has recently received increasing attention, especially topics related to DNA repair (Li et al., 2016) or glycolipid metabolism (Zhang et al., 2018).

Although all of these changes have occurred, studies investigating the genotoxic impact of Cr are still emerging, and Cr(VI) carcinogenicity is widely debated (Zhitkovich, 2011). In particular, the roles of non-coding RNAs in transcriptional responses during the exposure of physiological and toxicological levels are not well understood. Therefore, we conducted a toxicogenomics study using a data-driven analysis approach that aimed to outline integrated networks and to identify candidate key driver genes involved in the underlying mechanisms of cell cycle alterations after Cr exposure. Furthermore, considering the widespread involvement of long non-coding RNAs (lncRNAs) in multiple cellular functions (Mercer et al., 2009; Yao et al., 2019), including the cell cycle (Kitagawa et al., 2013) and other Cr-induced processes (Hu et al., 2019), we hypothesized that lncRNAs and coding genes might be key mediators of the responses to DNA damage by regulating the cell cycle in Cr-induced genotoxicity.

For the purpose of building a network to reveal the correlations between these RNAs, we integrated the results of several bioinformatic analysis approaches based on the expression data of RNAs. These included weighted correlation network analysis (WGCNA) to create modules according to highly correlated gene expression patterns (Langfelder and Horvath, 2008). Moreover, to pinpoint the key driver gene in these processes, we conducted weight key driver analysis (Shu et al., 2016) to extensively search for potential key elements in the regulation network (Sun et al., 2015; Bailey et al., 2018) and to detect the possible trigger genes in different phases of the cell cycle. Thus, our approach aimed to provide a candidate gene list for further research on explaining the underlying molecular mechanisms that regulate the cell cycle following exposure to relatively low concentrations of Cr(VI), and to identify the non-coding RNAs that might be novel candidate molecular targets for exposure biomarker studies.

MATERIALS AND METHODS

Cell Culture and RNA Extraction

The human bronchial epithelial cell line (BEAS-2B) was purchased from American Type Culture Collection (ATCC, United States), maintaining at 37°C and 5% CO_2 in a humidified incubator. Cells were cultured in Bronchial Epithelial Cell Growth Medium (BEGM^{TM}, BulletKit^{TM}Lonza, Switzerland) supplemented with the necessary components and growth factors. The BEGM media was replaced every second day, and cells were passaged when they reached 70–80% confluency by incubation with 0.25% trypsin. In the presence of a diluted potassium dichromate stock solution (K2Cr2O7, Sigma,

United States), BEAS-2B cells were seeded in six-well plates (105 cells/well) and exposed to low (0.2 μmol/L), medium (0.6 μmol/L), and high concentrations (1.8 μmol/L) of Cr(VI) for 24 h. A control group was established under the same conditions as the exposure groups. Each sample containing approximately 1×10^7 cells was disrupted in buffer RLT (Qiagen, United States) for RNA isolation. Total RNA was isolated using the miRNeasy Mini Kit (Cat#217004, QIAGEN, GmBH, Germany) according to the manufacturer's recommended guidelines, and the RIN number was determined to analyze RNA integrity using an Agilent Bioanalyzer 2,100 spectrophotometer (Agilent Technologies, Santa Clara, CA, United States). To assess the purity of RNA, a NanoDrop 2000c (Thermo Fisher Scientific, United States) and UVP Imaging System were used to measure the 260/280 ratios. Samples with a RIN number > 7 and 260/280 ratio in the range of $\sim$2.0 were considered qualified samples.

RNA Isolation and RNA Microarray

Total RNA was amplified and labeled using a Low Input Quick Amp Labeling Kit, One-Color (Cat.# 5190-2305, Agilent Technologies). Each slide was hybridized with 1.65 μg of Cy3-labeled cRNA with a Gene Expression Hybridization Kit (Cat.# 5188-5242, Agilent Technologies), maintaining in a Hybridization Oven (Cat.# G2545A, Agilent technologies) for 17 h hybridization. Then, slides were washed with the Gene Expression Wash Buffer Kit (Cat.# 5188-5327, Agilent Technologies) and scanned by an Agilent Microarray Scanner (Cat#G2565CA, Agilent Technologies). Finally, data were extracted with Feature Extraction software 10.7 (Agilent Technologies). Raw data were normalized with the quantile algorithm and limma packages in R.

Differential Expression Analysis

The normalization of gene expression and differentially expressed (DE) gene analysis were performed by functions in R package limma (Ritchie et al., 2015). Briefly, the function lmFit was used to fit a linear model to estimate the variability in the data. Using the function eBayes in limma, the significance of differences in the variance of gene expression across biological replicates for each gene were calculated using empirical Bayes moderated t-statistics tests. For the multiple hypothesis testing correction, the false discovery rate (FDR) was applied. The fold change in logarithm form between the samples from treated group and the samples from control group was also calculated for each gene (Smyth, 2005). Finally, genes were defined as DE if FDRs were below 0.05 and $|\log2FC| \geq 1$.

Cell Cycle-Related Genes

After analyzing the pathways, significantly expressed pathways were identified with the cutoff of adjusted $P < 0.05$. From our results, we observed that significant pathways were related to biological processes involved in the regulation of the cell cycle, which had 11 related pathways. Furthermore, we extracted all genes with the GO annotations of the cell cycle (GO:0007049) from AmiGO 2 (Carbon et al., 2009) and withdrew the DE genes in our database. At last, a linear regression was performed with the expression of each gene. We used the 12 values in 4 dose groups (each dose had triplicated biological samples) as the dependent variable, and the dose 0–1.8 μmol/L corresponding to each sample as the independent variables. We took the linear regression slope to represent expression-dose-depend relationship for each gene and filtered the genes with a threshold of the median coefficient from the whole gene set. The g:Profiler (Reimand et al., 2016) (database built on 2020-03-07) was used to conduct GO enrichment analysis based on the DE gene list identified from the high dose group vs. with the control group comparison. We also visualized the g:GOSt (database built on 2020-03-07) enrichment results from different annotation resources, including GO, KEGG, REAC, and TF.

Co-expression Network Detection Using WGCNA

WGCNA (Langfelder and Horvath, 2008) was used to identify the co-expression module with the selected cell cycle-related coding RNAs. WGCNA is a guilt-by-association approach for constructing networks and module detection. We computed a correlation raised to a power as a soft thresholding between every pair of RNAs to amplify the disparity and transformed the result into an adjacency matrix. Then, the blockwiseModules function was used to compute topological overlap matrix (TOM) dissimilarity (Yip and Horvath, 2007) between genes, and a hierarchical clustering gene dendrogram was constructed. According to the standard of dynamicTreeCut, modules whose eigengenes are highly correlated are merged with the threshold of 0.2. At last, we applied the module preservation analysis to test whether the features of each module were preserved in an alternative set of samples.

The preservation analysis is to estimate the differences between the observation in our gene expression and random situations by permutation (nPermutations = 10). Furthermore, Z-summary statistical analysis was performed as a general summary of all of the different statistics used, and scores > 10 were considered preserved (Langfelder et al., 2011).

Key Driver Analysis

To conduct key driver analysis, we used the R package KDA (v1.14.0) (Shu et al., 2016). The purpose of KDA analysis was to analyze the detailed interactions between coding genes and lncRNAs to select genes that were over-represented in the regulation of the cell cycle. The package first required a sub-network file and gene list of interest as input files. Then, the enrichment in the k-step downstream neighborhood for the target gene list was assessed in the sub-network. In this study, we used a list of cell-cycle related genestarget list as the input file. We computed the co-expression pairs between targeted coding genes and all lncRNAs in our experimental study as the network file. We divided these coding genes into three group according to the key events in distinct phases, for example the checkpoint which regulated the transition of phases. Finally, we combined the lists with the name of cell cycle class as the input lists for KDA detection. The KDA results were visualized using Cytoscape (v3.7.2).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For quantitative real-time PCR (RT-qPCR) analyses, total RNA was isolated from the control, middle, and high dose groups of the samples as described above, and those groups along with three independent replications of the biological experiments were analyzed. We selected and verified the key driver genes and genes in the network (Figure R1) for their biological potential in explaining the Cr(VI) cytotoxicity with the following criteria: (1) the genes participate in regulating at least two stages of cell cycle progress in the subnetwork; (2) the genes were reported to be involved in the biological functions, such as cell cycle arrest, carcinogenicity, and lung cancer, according to the literature. According to these criteria, we selected three groups of genes, in the (1) PARD3 group: ENST00000607815 and PARD3B, (2) SND1group: TEX14, (3) genes in both PARD3, and SND1 group:lnc-NCS1-2:1, FBXO6, CDK14, lnc-DHX32-2:1, lnc-FOXK1-4:1, POLA1, NR_002579, lnc-C3orf14-1:1 as the target genes for further validation.

Primer sequences were designed using Primer Express 3.0.1 designer software and then verified with NCBI Primer-BLAST software to confirm specific recognition of the target lncRNAs and mRNAs. qPCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, United States) to determine the expression levels of selected genes, and the results were presented as the mean value for the three wells. Data were calculated using the $2^{-\Delta\Delta Ct}$ with glycerol-3-phosphate dehydrogenase (GAPDH) as the endogenous control.

RESULTS

DE Genes Between Different Groups

In total, we collected 12 samples, 3 from the low-dosage group, 3 from the medium-dosage group, 3 from the high-dosage group and 3 from the control group. Genome wide analysis of RNA expression by microarray was used to examine the transcription changes between the Cr(VI) treated group and the control group. To fully characterize the regulation pattern between mRNA and lncRNA, 18833 mRNA and 68104 lncRNA were considered in this study. Differential expression analysis was conducted for each Cr(VI) dosage vs. control comparison. The largest differences in gene expression were evident in the high dose group (1.8 μmol/L) with log2 fold-change [log2(FC)] values ranging from −3.76 to 4.52, including 646 upregulated and 771 downregulated genes (**Figures 1B,D**). A relatively lower number of differentially expressed genes was observed in the middle (129) (**Figures 1A,C**) and low dose (17) groups compared with normal cultured cells. It was evident that there were more upregulated and downregulated lncRNAs in the 1.8 μmol/L group compared with the other groups (**Figure 1C**), and DE lncRNA from high-dose accounts for 89.3% of all DE lncRNAs across three groups. From the histograms of fold change profile for mRNA and lncRNA in the two groups, we found that the high dose group had more differentially expressed transcripts with log2FC values above 0.5 and below −0.5 than the middle dose group (**Supplementary Figure 1A**), whereas the differences in lncRNAs were not as clear (**Supplementary Figure 1B**). Taken together, the high level of hexavalent chromium induces the dramatic changes in transcription profiling. To get a unified DE gene set, we merged the DE genes from three different groups and 1,484 mRNAs and lncRNAs were left (**Supplementary Table 1**), while the detail information was listed in **Supplementary Tables 2**, **3**.

Enrichment of Protein-Coding Genes

To further explore the potential biological processes induced by Cr, enrichment analyses using DEGs databases were conducted using g:Profiler. As shown in **Figure 2**, DNA replication (FDR = 5.97×10^{-18}) and chromatin organization (FDR = 2.70×10^{-12}) from GO biological process database, cell cycle-related pathways from the Reactome Pathway Database (REAC) were identified with almost the highest statistically significant confidence level (all FDR < 0.05), suggesting that these processes might substantially change after exposure to the Cr(VI) (**Figure 2B**). Moreover, after analyzing the most altered pathways in replicating cells in detail, we identified the pathway involving SIRT1 (FDR = 4.56×10^{-22}), a component of the Energy-dependent Nucleolar Silencing Complex (eNoSC), which may serve as an important integration point in cell cycle regulation (Bouras et al., 2005). Other pathways, including DNA double-strand break repair (FDR = 0.017) and mitotic cycle alternation (FDR = 0.046), also suggested a link between the modification of the cell cycle and exposure to Cr(VI). Therefore, we selected genes with strong enrichment with specific processes as the target biological reactions for analysis.

Selection of Cell Cycle-Related Genes

To determine the gene list for further analysis of transcript profiles and co-expression with lncRNAs, we select cycle-related genes based on three criterion: (1) genes annotated with the function of cell cycle, (2) DE genes, (3) existing linear relationship. We first downloaded the coding gene data and their information regarding GO class and evidence code from AmiGO 2 with the annotation of the cell cycle (GO:0007049) as the background list. Subsequently, 64 gene transcripts were matched with our differentially expressed genes, and the list in AmiGO 2 of 918 genes was related to 1,952 items with target processes. These genes were then screened according to the criterion for dose-expression relationships. To accurately identify the genes related to the effect of Cr(VI) treatment on cell proliferation, we filtered genes with absolute regression coefficients > 0.4 (close to the value of Q1 and Q3 in the cell cycle). Finally, 35 genes were selected as targeted mRNAs for the construction of co-expression modules with lncRNAs.

The expression levels in the four treatment groups from the mRNA profiles and evidence codes of these genes in the cell cycle are shown in **Figure 3**. As shown in **Figure 3A**, genes were clustered into four groups, even with similar pathways in the process of regulation of the cell cycle in all clusters, we still found the cluster-specific pathway, indicating that Cr(VI) may affect the regulatory functions in different aspects. In particular, cluster one was enriched in the function of cell division ($P = 1.16 \times 10^{-6}$),

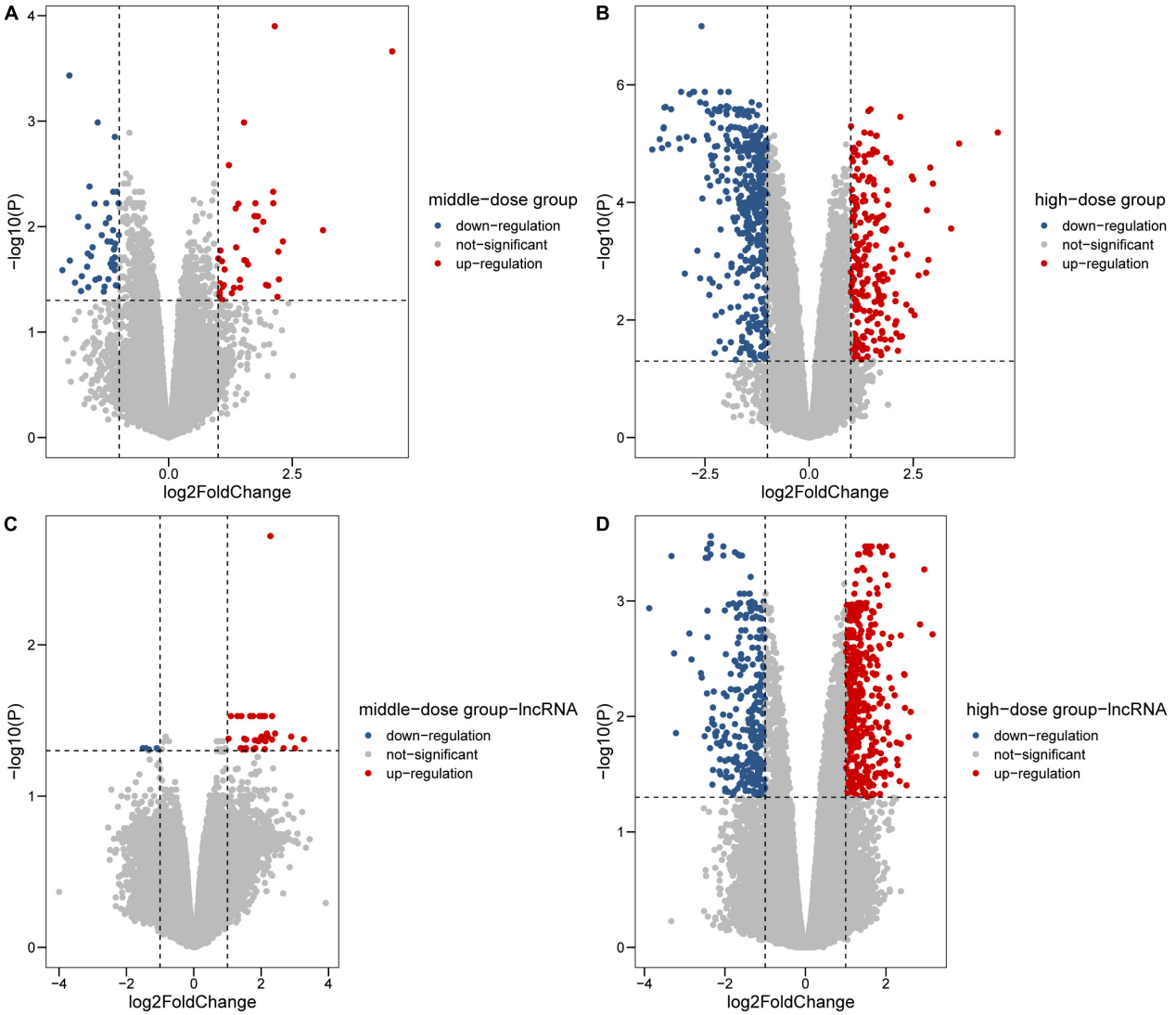

FIGURE 1 | Differentially expressed genes in 0.6 and 1.8 μmol/L Cr(VI) groups. **(A,B)** Differentially expressed mRNAs in 0.6 and 1.8 μmol/L Cr(VI) groups. **(C,D)** Differentially expressed lncRNAs in 0.6 and 1.8 μmol/L Cr(VI) groups.

whereas cluster two focused on mitotic cell cycle phase transition ($P = 6.11 \times 10^{-6}$), especially the G2/M transition of the mitotic cell cycle ($P = 2.30 \times 10^{-4}$). The last cluster with 9 genes, including IL1A and IL1B, was associated with the regulation of cell cycle processes ($P = 3.76 \times 10^{-5}$). Additionally, as shown in **Figure 3B**, 17 of 35 gene annotations were derived from laboratory experiments, suggesting that the transcripts were closely related to the process of cell cycle regulation.

Co-expression Module With Cell Cycle-Associated Protein-Coding Genes and lncRNAs

To investigate lncRNAs that were highly connected to a given set of interesting protein-coding genes, WGCNA was employed. WGCNA is R software package to perform network construction, module detection, and calculation of topological properties based on the guilt-by-association strategy. The WGCNA network was generated using the selected 35 mRNA and all lncRNA profiles from the different dose groups. We selected 16 as the soft threshold based on the results of scale-free topology and mean connectivity for the construction of the network. All genes were clustered into a unique module and the cluster dendrogram shown in **Supplementary Figures 2, 3**. Altogether, we identified six modules that contained target cell cycle protein-coding genes from all 185 co-expression modules. Modules were color-coded as brown, plum1, black, gray60, magenta, and tan4 with 25, 5, 2, 1, 1, and 1 mRNA, respectively. A preservation test was applied to confirm the reliability and sensitivity of the results using a WGCNA function (modulePreservation). Briefly, to evaluate whether a module was conserved or not, the Zsummary (Z-score) was calculated. All six modules were highly preserved with

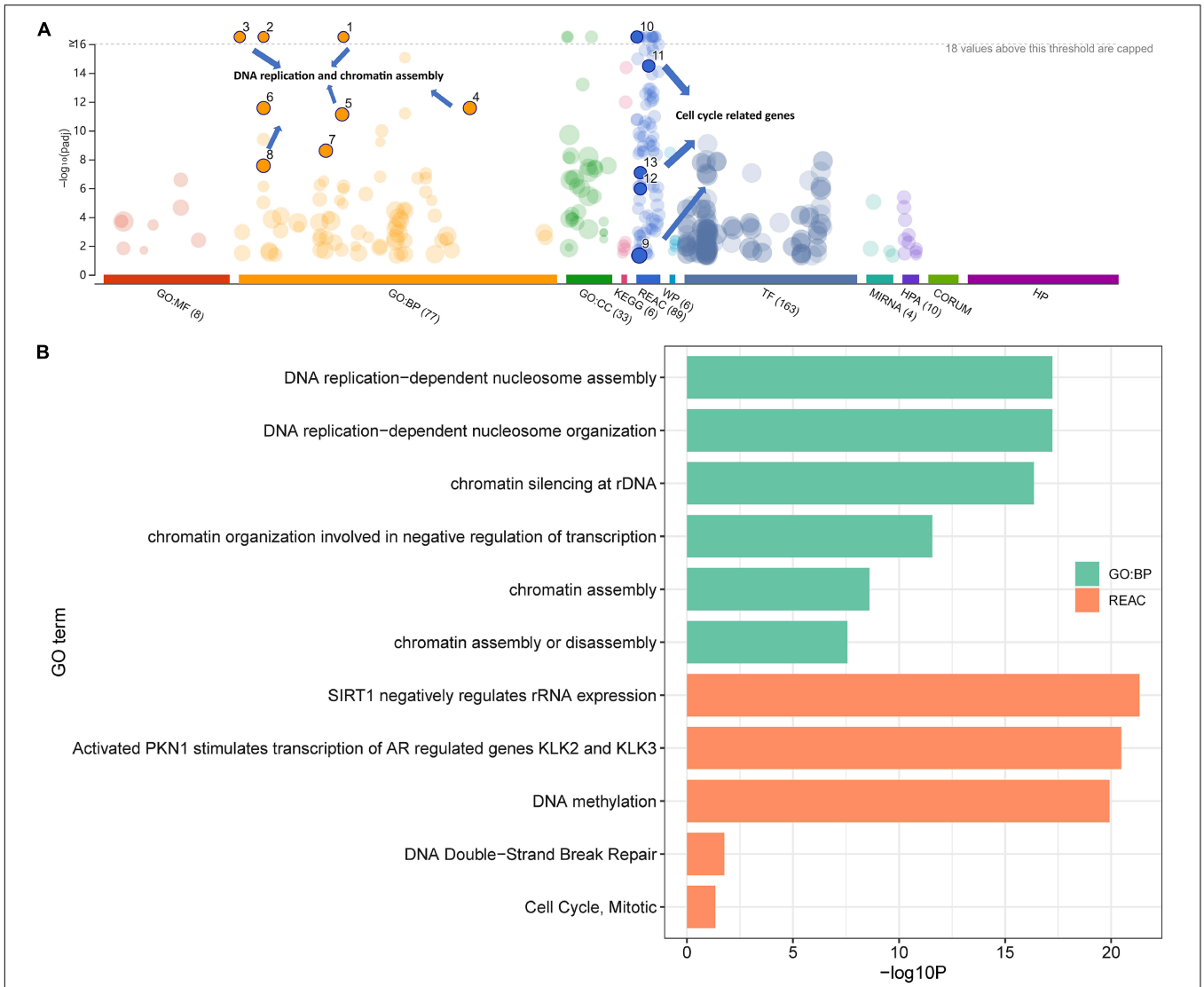

FIGURE 2 | Enrichment analysis of differentially expressed genes in the high-dose group. The total transcript level correlates to **(A)** cell cycle processes and **(B)** detailed information for the chosen pathways.

Z-scores > 30, whereas the brown ($Z_{summary}$ = 68) was chosen for obtaining the highest number of transcripts (**Figure 4**). Therefore, modules colored brown containing 25 mRNAs and 3,565 lncRNAs were regarded as highly representative modules according to the expression patterns of these genes, and served as a cell cycle-regulated network for further analysis.

Key Driver Analysis

Previous analysis recommended that genes Within the brown module Were the key cell cycle regulatory gene set. We performed key driver analysis (KDA) for these target gene co-expression modules using our previously published method (Shu et al., 2016). Key driver genes or key regulatory components were defined as the subnetworks with respect to various biological contexts, when compare to the other random genes in the network. The KDA requires a gene list (generally a biological-associated gene list) and a gene network as input files to identify the key genes. Under different cell cycle phases, we first divided 25 mRNA in the brown module Into three categories (G1/S, G2/S, and M) according to their GO subcategory annotation (with the highest level of evidence code in each gene), which were connected to the biological regulatory events in cell cycle processes. Then, Pearson's correlation coefficient was calculated to each selected protein-coding gene and all differentially expressed lncRNAs. Pairs with absolute values of Pearson's correlation coefficients ≥ 0.90 were selected as the network file.

Finally, three top key driver genes, including PARD3 (FDR = 1.46×10^{-22}), SND1 (FDR = 5.24×10^{-8}), and lnc-DHX32-2:1 (FDR = 1.43×10^{-17}), were particularly highlighted in the category of G2/M, G1/S, and M phases

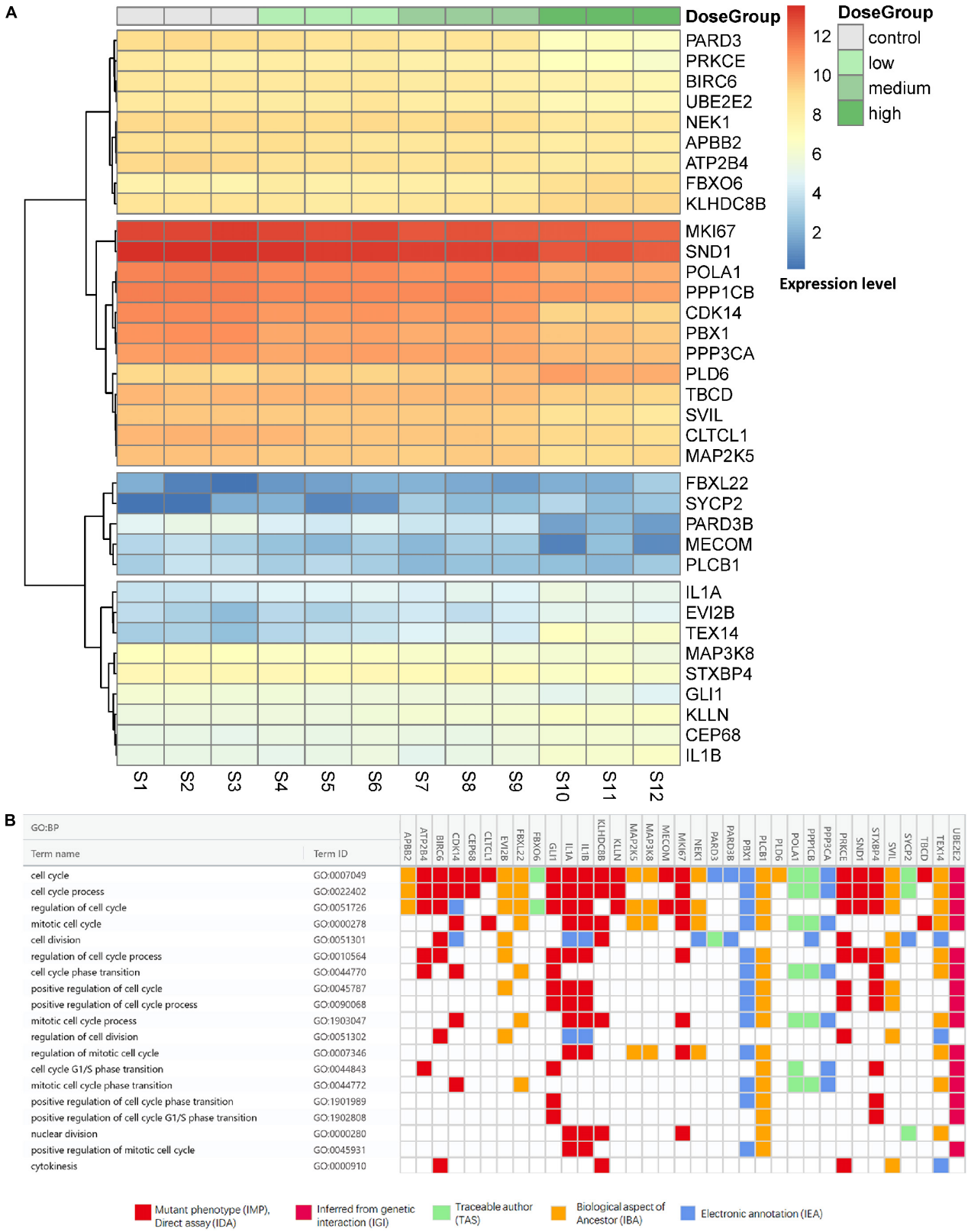

FIGURE 3 | Expression levels and evidence codes of target cell cycle genes. **(A)** The expression level of the cell cycle-related genes in different groups and samples and **(B)** the gene ontology evidence codes of the selected genes in the cell cycle.

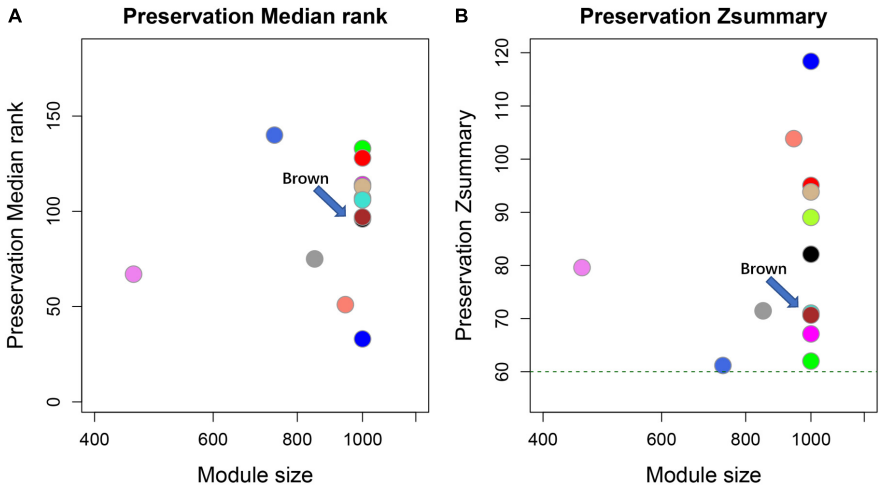

FIGURE 4 | Module preservation test for co-expression cell cycle mRNA data sets. Circles in both figure represent different co-expression modules. The horizontal and vertical axes represent gene number and $Z_{summary}$ values for each module with random selection, respectively. The green dotted line indicates $Z_{summary} = 60$ ($Z_{summary} > 10$ indicates high preservation). **(A)** Preservation median rank of these modules. **(B)** Preservation $Z_{summary}$. Brown modules were indicated by arrows.

and mathematically identified as the causal modulators of the regulatory state of the functionally relevant gene group based on prior knowledge (**Figure 5**). Moreover, several genes showed great connectivity in our causal gene network and interrelated with every single key driver gene, including CDK14, POLA1, lnc-NCS1-2:1, and lnc-FOXK1-4:1 (all FDR < 0.05 in those phases). Furthermore, lnc-DHX32-2:1 and PARD3B existed in three categories in our list, illustrating their potential relationship within the probabilistic causal gene network, which might suggest their complex cellular context. Additionally, some significantly differentially expressed genes existed in the network, such as FBXO6 and ENST00000607815. It was also revealed that most genes might participated in multiple phases regulation, especially in the progression G1/S and G2/M phases.

Validation by Real-Time RT-PCR

To determine whether the expression patterns of these genes could be recapitulated, we selected ENST00000607815, lnc-NCS1-2:1, FBXO6, CDK14, lnc-DHX32-2:1, lnc-FOXK1-4:1, PARD3, PARD3B, POLA1, SND1, NR_002579, TEX14, lnc-C3orf14-1:1 for real-time RT-PCR analysis (**Figure 6**). The results found 11 different expressed genes in our network, including the key driver genes SND1 and PARD3, which had significantly change between control and 1.8 μmol/L group. Eight genes which both connected with PARD3 and SND1, including lnc-NCS1-2:1, FBXO6, CDK14, lnc-DHX32-2:1, lnc-FOXK1-4:1, POLA1, NR_002579 and ENST00000607815 could be divided into three groups, (1) six of them located in G1/S and G2/M phase sub-nodes, (2) lnc-DHX32-2:1 related with all cell cycle phases change, and (3) FBXO6 was in G2/M or M phase node. Among these genes, lnc-FOXK1-4:1, POLA1 and ENST00000607815 had the most significant change in Cr group, indicating the potential effect on G1/S and G2/M phases.

DISCUSSION

The present study provided a regulatory network containing protein-coding genes and lncRNAs, including PARD3, SND1, POLA1, FBXO6, lnc-NCS1-2:1, lnc-FOXK1-4:1, lnc-DHX32-2:1, and ENST00000607815, which revealed the key regulatory mechanisms in different cell cycle phases after exposure to Cr(VI). Because our *in vitro* experiment was based on relatively low doses of Cr, it can be referenced as a common environmental exposure level that mimics the damage on human respiratory epithelial cells. Our results regarding the cell cycle were consistent with previous findings that Cr exposure could induce cell cycle changes by regulating checkpoint pathways to control the order and timing of cell cycle phase transition (Alcedo et al., 1994; Chiu et al., 2010; Nickens et al., 2010; Proctor et al., 2014; Rager et al., 2019), even under very low concentrations (Ha et al., 2004; Wakeman et al., 2004). In particular, the bioinformatics approach we used provided us with potential candidate lncRNAs that might play important roles in Cr(VI) exposure via the regulation of different cell cycle phases.

In the past two decades, the number of papers related to the cell cycle has rapidly increased (Kastan and Bartek, 2004; Malumbres and Barbacid, 2009) because of the complex and diverse processes involved in the cellular responses to DNA damage. Both endogenous and exogenous sources that cause DNA damage are considered major contributors in the development progress of human cancers, and thus it is reasonable to speculate that defects in cell cycle genes have critical significance in increasing the respiratory cancer risk for occupational exposure to Cr(VI) (Seidler et al., 2013). Previous transcriptomic-based studies have indicated that genes relevant to cell proliferation and DNA repair showed differential expression patterns after Cr(VI) exposure. Among these pathways, the effects of inhibitors of ATM activation have received the most attention because of the observed toxicological

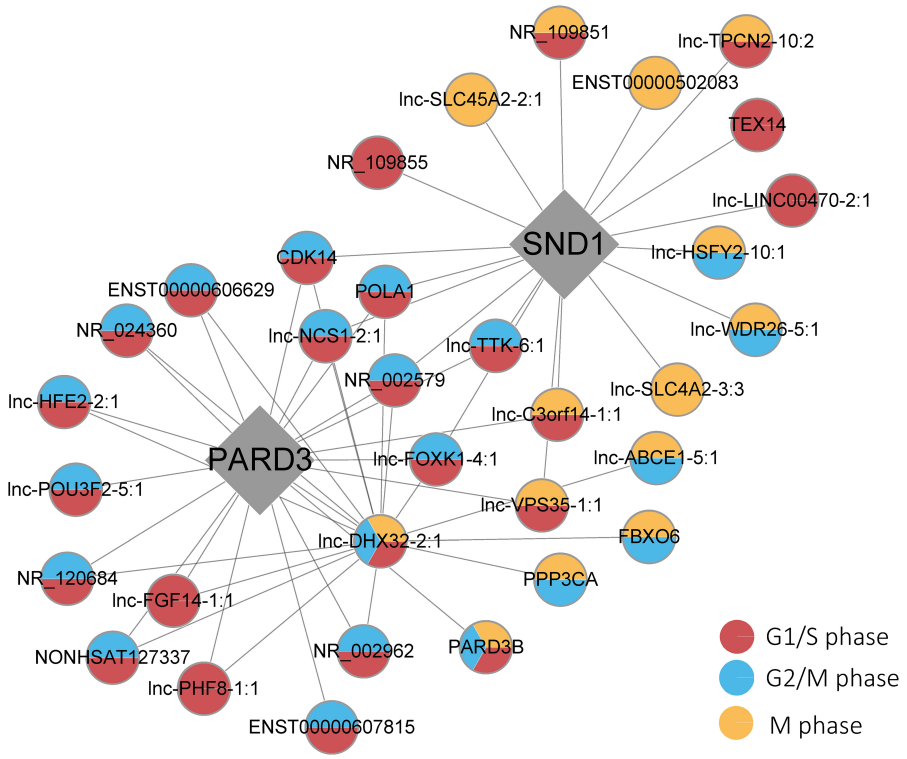

FIGURE 5 | Key driver gene subnetworks with the phases of the cell cycle. Red, blue, and yellow circles indicate G1/S, G2/M, and M phases, respectively. Diamonds represent the key driver genes.

importance in activation of the DNA damage-responsive kinase ATM after Cr(VI) exposure (Luczak et al., 2016).

However, this pathway did not appear in our experimental study, which may be because of the threshold of checkpoint activation or DNA-damage response (Liang et al., 2014). Our results offered novel candidate regulatory genes using mathematical algorithms and cells treated with relatively low doses (< 2 μmol/L) of Cr(VI), which altered the cell cycle phases in a concentration-dependent manner. The high level of complexity in cell cycle regulation after exposure to potassium dichromate provides a great opportunity to discover novel factors in several signaling and response pathways to address the specific nature of cell damage. To minimize the adverse effects of DNA damage situation, DNA repair is a mechanism that allows cells to properly repair these defects. This is exemplified in the results of a study involving PARD3 and PARD3B, which encode proteins belonging to the Par-3 family of cell polarity regulators, that play key roles in asymmetrical cell division and polarized cell growth. Studies showed that Par-3 complexes could regulate DNA-PK directly or via Ku70 which could affect the progress of DNA double-strand break repair (Fang et al., 2007), thus indicated that Par-3 enhanced NHEJ and HR pathway required for efficient DNA repair in G2 and M phases. Besides, the loss or attenuation of epithelial polarity is a hallmark of epithelial to mesenchymal transformation (EMT) (Thiery, 2003), indicating the possibility of regulating cell cycle and enhancing the genetic instability by PARD3 or PARD3B (**Supplementary Figures 2, 3**). Together, this suggests the possibility that Par-3 is involved in cancer development and progression in the G1/S or G2/M phase. Moreover, the gene SND1 (staphylococcal nuclease and tudor domain containing 1) in the center of the network mediated miRNA decay of both protein-free and AGO2-loaded miRNAs and also regulated mRNAs involved in G1-to-S phase transition (Elbarbary et al., 2017). Extensive research also supports the conclusion that SND1 is an oncoprotein in a variety of cancers involving multiple processes (Jariwala et al., 2017; Xin et al., 2019) and that it also acts as an essential effector in promoting EMT in cancer (Xin et al., 2019; He et al., 2020). In addition, FBXO6 was found to closely interact with Chk1 (Tu et al., 2017), which indirectly affected the ATR-Chk1 signal axis by various kinds of DNA damage insults, including replication stress, inter-strand cross-link (ICL), and DSBs (Jazayeri et al., 2006). Similarly, a study demonstrated that FBXO6 correlated with the survival of non-small cell lung cancer (NSCLC) patients (Cai et al., 2019). CDK14 plays fundamental role in regulating the G2/M phase of the cell cycle by mediating the phosphorylation of LRP5/6 in the Wnt signaling pathway (Wang et al., 2016). Correspondingly, CDK14 played an important role in lung tumorigenesis. For example, the cigarette smoke-induced downregulation of CDK14 in lung cells correlated with β-catenin levels, suggesting impaired Wnt signaling (Pollack et al., 2015). Another study also suggested that CDK14 is regulated by SNHG15 by competitively sponging miR-486, which contributed to NSCLC tumorigenesis (Jin et al., 2018).

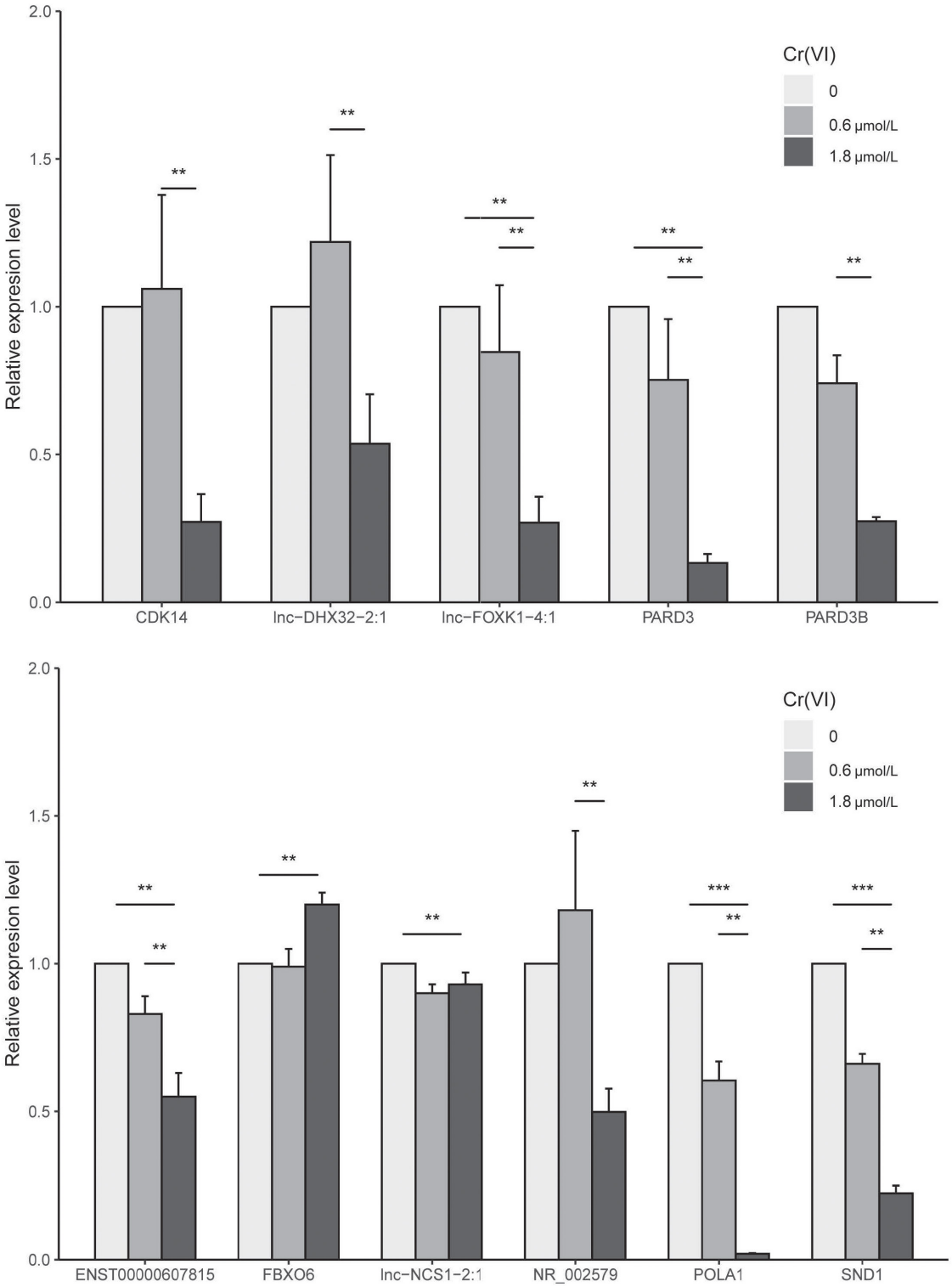

FIGURE 6 | Results of RT-PCR analysis of 11 genes in the network. ** indicates significantly different at 0.05, *** indicates significantly different at 0.001.

As RNAs do not function alone, we conducted WGCNA and probabilistic causal gene analyses to model underlying molecular relationships and causal gene connections, and used mathematical approaches and key driver analysis (KDA) to prioritize key drivers of the genotoxicity of Cr(VI).WGNCA analysis was conducted to narrow down

the extent of related lncRNAs with our selected genes with a dose-response relationship, and KDA was used to identify the key regulator within the co-expression network lncRNAs, including ENST00000607815, lnc-NCS1-2:1, lnc-DHX32-2:1, and lnc-FOXK1-4:1, were verified as the important lncRNAs in our network. One of the main regulators of lnc-DHX32-2:1, which targeted for gene ADAM12, has been implicated in a variety of biological processes, including lung cancer and the development of giant cell tumors, and positively involved in the regulation of the MAPK/ERK pathway. Moreover, the MAPK/ERK pathway plays an important role in integrating external signals to promote the transition of G1 to S phase, while ERK activation downstream of mitogen-induced Ras signaling is sufficient to alleviate cell cycle arrest and allow cells to progress to the S-phase (Chambard et al., 2007). This is consistent with a previous study in which Cr(VI) activated ERK signaling pathways (Chuang et al., 2000), although no cytotoxicity effects were observed. Indeed, our analysis identified lncRNAs as interesting candidates involved in cell-cycle gene regulation, but all guilt-by-association approaches must be treated with caution and recognized as hypothesis-generating research. Additionally, all exploratory data analysis techniques require extensive targeted studies to confirm suggested molecular networks and the potential mechanisms.

Finally, our study revealed that cells exhibited cytotoxicity even in the low-dose exposure group. The choice of relatively low concentrations based on the conclusion of previous studies showed that BEAS-2B cells exhibited increased proliferation in the presence of 1 μM (Nickens et al., 2010; Cerveira et al., 2014), whereas other studies showed slightly decreased proliferation following treatment with low micromolar range concentrations after stimulating human bronchial epithelial cells (Abreu et al., 2018; Hu et al., 2019). The 2 μM concentration was considered to cause significant cytotoxic effects and alterations of the cell cycle. Therefore, increasing the sample size and number of doses would allow for more convincing conclusions. Our research design follows the principle of Toxicity Testing in the 21st Century, which emphasized the usage of cell lines, computational modeling, and bioinformatics approaches to explain cell response progress (Krewski et al., 2010; Seidler et al., 2013). This research also implied that the DNA repair or immune defense mechanisms might involve greatly to cope with the toxicity in relatively low level, which is different from the proved results, and can help assess the dose-response characteristics in exploring the perturbation of the adverse pathways (Krewski et al., 2010).

The current study had several limitations. First, the use of single omics technology reveals the cellular transcription responses patter on Cr, which might have limitation in explaining the results in the aspect of functional outcomes. Subsequently, the transcriptome experiment conducted by microarrays with high background levels owing to cross-hybridization confines the accuracy of expression estimation, particularly for the transcripts in low abundance (Zecevic et al., 2009). Finally, this study based on a single cell line that cannot depict the whole picture of complicated toxic responses in human pulmonary bronchial. Thus, further research on different human cell as well as the *in vivo* approaches, such as cell cycle checkpoint and related protein analysis, that aiming to test the regulation of cyclin-dependent kinases secretion with knock-out amplification these genes can offer evidence on how they activate or inhibit cell cycle phases, which can be used to explain the carcinogenicity and Cr(VI) toxicity. Another potential limitation is that repeated experiments under different passages and freeze downs are needed for further analysis. In the future, we will apply this method to other cell lines, for example, HBECs and hTERT-immortalized lung Cells. With the development on 3D-cell-culture model *in vitro* (Gunness et al., 2013; Amann et al., 2014). Our further research will try to analysis the transcriptome character in different condition with the 3D Cultures method to construct lung model (Rayner et al., 2019). Nonetheless, further integrated proteomics, epigenomics or the RNA modifications analysis on multi-omics level and time series studies with RNA-seq methods are needed to confirm the mechanisms of toxicity involved in the cell cycle alterations induced by Cr(VI).

CONCLUSION

We designed a novel computational workflow that showed the toxicity effects of Cr(VI) on the cell cycle. We identified gene networks and candidate lncRNAs and mRNAs in relatively low dose range suggesting that the gene sets could provide a clue for toxic responses in cell cycle regulation to environmental and occupational Cr(VI) exposures.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/reefur/Chromium-transcriptome/blob/main/database_cr.xlsx.

AUTHOR CONTRIBUTIONS

PZ and ZW participated in the study design, statistical and bioinformatics analysis, and manuscript preparation. SH, HF, and FH conducted the experiment. DZ and CL provided assistance with gene set network analysis. GH, ZC, ZW, TW, and GJ reviewed the figures and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.597803/full#supplementary-material

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Systematic Review of Potential Occupational Respiratory Hazards Exposure Among Sewage Workers

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Background: Sewage workers have a higher risk of exposure to various potential occupational respiratory hazards found in sewage plants. Although previous studies discuss occupational respiratory hazard concentration impacting sewage workers' respiratory health, the results are scarce and mixed. Hence, there is a need to identify the potential respiratory hazards in sewage plants so as to clarify the short- and long-term respiratory health effects. Therefore, this systematic review (SR) aims to critically review previous studies investigating potential respiratory hazards found at sewage plants and their effects on sewage workers' respiratory health.

Methods: An SR was conducted using PubMed, EBSCO Medline, Web of Science, Scopus, and Google Scholar on peer-reviewed studies published between January 1994 and October 2020 evaluating the impact of potential exposure to respiratory hazards and its effects on respiratory health among sewage workers. "Sewage treatment plant," "respiratory hazards," and "respiratory health effects" were the three main search terms chosen in this SR. The inclusion criteria were (1) studies on potential occupational respiratory hazard exposure among sewage workers, (2) manuscripts written in English, and (3) studies published in the peer-reviewed literature. The human observational studies' quality was assessed using the Effective Public Health Practice Project Quality Assessment Tool.

Results: We identified 5,660 articles through an initial database search. Only 26 items met the inclusion criteria and were included in this review; 15 human observational studies and 11 environmental assessment studies were conducted in the sewage industries. Most of the human observational studies were rated as moderate quality, two studies were rated as weak quality, and one study with strong quality was identified. Hydrogen sulfide, bioaerosols, particulate matter 2.5 (PM 2.5), and volatile organic compounds (VOC) were found to be potential respiratory hazards. Most of the risks contributed to adverse outcomes on the sewage workers' respiratory health with some inconsistent findings on the relationship between respiratory hazard exposure and respiratory health effects.

Conclusion: Our review finds that, although this area is of great importance, quality studies are still lacking. There is a need for additional studies to clarify the effects of respiratory hazard exposure on sewage workers and respiratory health, especially PM 2.5 and VOC.

Keywords: respiratory hazards, sewage workers, sewage plants, respiratory symptoms, pulmonary function

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INTRODUCTION

Various studies widely demonstrate that sewage treatment plants (STP) produce bundles of occupational hazards through different sewage plant processes to remove contaminants from wastewater or as by-products (1, 2). Occupational hazards can be described as "aspects of one's occupation-specific context that increase the risk of injury" (3). Occupational hazards refer to the potential risks to the health and safety of those people who work outside the home. Generally, there are several occupational hazards that potentially exist in the sewage plant, such as chemical, biological, and physical hazards (4). Exposure to these potential occupational hazards can lead to work-related diseases and adverse health effects. As a result, much previous literature shows that workers at sewage treatment plants are at high risk of experiencing a broad range of adverse health impacts, including respiratory disorders (asthma or chronic obstructive pulmonary disease), infections (such as tuberculosis, leptospirosis, hepatitis A, or tetanus), gastrointestinal problems (for example, gastroenteritis), skin illnesses (for instance, contact dermatitis or eczema), cancers (such as lung, stomach, and renal cancers), and general symptoms (such as unusual tiredness and headache) (4–6).

Occupational lung diseases are among the leading health impacts because sewage workers are likely to be exposed to various occupational respiratory hazards ranging from specific chemical agents to microbiological agents (7). Generally, occupational respiratory hazards can be present in several forms at any industry's workplaces, for instance, gases, dust, fumes, mists, vapors, smoke, fog, and sprays. Some substances are generated via industrial processes, for example, those tailored to sewage industries, such as during the aeration process, drying the sludge, and mechanical filtering processes. Sewage workers exposed to occupational respiratory hazards for a significant amount and duration can develop adverse respiratory health effects without proper hazard control strategies and personal protective equipment (8). These are usually due to infection, inflammation, and chemical sensitization along the airway tract and allergic responses (9).

To date, occupational lung diseases have become a global issue and have been researched extensively as they act as a significant contributor to morbidity and mortality. Based on the Global Burden of Disease Study (10), occupational respiratory hazard exposure is a crucial determinant of chronic, work-related respiratory disease. Also, it accounted for more than 500,000 mortality incidents and 13 million disability-adjusted life years in 2016 from chronic respiratory disease due to occupational respiratory hazard exposure (10). Besides that, the American thoracic society finds that the highest number of occupational diseases frequently recorded among sewage workers were respiratory (66%) followed by skin problems (31%) and noise-induced hearing impairment (11, 12).

Nowadays, the development of sewage plants has resulted in new technologies. Different processes of treating wastewater could contribute to further production and concentration of toxic air pollutants (13, 14). Even though several previous studies measured occupational respiratory hazard (i.e., hydrogen sulfide, endotoxins, inhalable dust) concentrations and the exposure effects on sewage workers' respiratory health, the results are scarce and mixed. The potential impacts of mixed gas exposure and dose-related effects could attenuate this issue further.

Objectives

As such, the purpose of this systematic review is to identify potential occupational respiratory hazard exposure among sewage workers that could arise from sewage treatment plants and its effects on sewage workers' respiratory health with the intention that, in the future, this review may provide input to the authorities to plan several strategies to prevent and minimize as much as possible sewage workers' respiratory health effects from the identified occupational respiratory hazard exposures. This review extensively examines published studies of potential occupational respiratory hazard exposure among sewage workers and its impact on their respiratory health. Thus, this review includes articles from human observational studies examining the respiratory health effects of potential occupational respiratory hazard exposure. Environmental assessment studies are also included to measure the occupational respiratory hazard concentrations produced at sewage plants.

Research Question

The key question of interest is the following: What are the potential occupational respiratory hazards that can be found at sewage plants and their effects on sewage workers' respiratory health?

METHODS

Eligibility Criteria

A broad systematic review was conducted based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. We developed several eligibility criteria to address these critical question: What evidence of potential occupational respiratory hazards could arise from the sewage treatment plant and possibly cause adverse respiratory health effects among sewage workers? To widen our findings, we selected (i) observational studies involving human subjects and (ii) environmental assessment studies conducted in sewage plants. Both types of studies need to be undertaken in the sewage industry settings. The inclusion criteria were (i) studies on potential occupational respiratory hazard exposure among sewage workers, (ii) manuscripts written in English, and (iii) studies published in the peer-reviewed literature from January

Abbreviations: CNS, Central nervous system; COPD, Chronic obstructive pulmonary disease; DALY, Disability-adjusted life years; EPHPP, Effective Public Health Practice Project; ELISA, Enzyme-linked immunosorbent assay; FEV%, Forced expiratory volume percent of predicted value; FEV1, Forced expiratory volume in 1s; FVC, Forced vital capacity; HAdv, Human adenovirus; H_2S, Hydrogen sulfide; HCHO, Formaldehyde; NH_3, Ammonia; NO, Nitric oxide; NO_2, Nitrogen dioxide; PCR, Polymerase chain reaction; PEF%, Peak expiratory flow percent of predicted value; PICO, Population, Intervention, Comparison and Outcomes; PM 2.5, Particulate matter 2.5; PPE, Personal protective equipment; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; QMRA, Quantitative Microbial Risk Assessment; SO_2, Sulfur dioxide; SR, Systematic review; STP, Sewage treatment plant; VOC, Volatile organic compound; WWTP, Wastewater treatment plant.

1994 to October 2020. Meanwhile, the exclusion criteria were in the categories of (i) review articles and (ii) case report or forensic studies.

Search Strategy

Comprehensive database searches were conducted between May and October 2020. All the relevant kinds of literature were extensively searched via five databases: PubMed, EBSCO Medline, Web of Science, Scopus, and Google Scholar. The search strategy was based on implementing the population, intervention, comparison, and outcomes (PICO) model developed first for MEDLINE and later adapted for other databases. The PICO approach utilized to guide us in constructing the organization and syntax of search terms was as follows: For population, we only selected sewage workers and studies done in sewage plants. For intervention, no interventional studies were expected in this study area given the observational nature of potential occupational respiratory hazard exposure among workers in sewage plants. For control/comparison, any comparison method could be included; it was not limited or specified provided the specific details of potential occupational respiratory hazard exposure monitoring and/or respiratory health effects among the study participants were reported. For outcomes, we looked into sewage workers' respiratory health effects from the exposure of potential occupational respiratory hazards that exist in sewage plants. Thus, the main search terms chosen were sewage treatment plant, respiratory hazards, and respiratory health effects. We used several search terms, such as "sewage treatment plant" well-defined with synonym "wastewater treatment plant." Next, for the terms of respiratory health effects, we included several search phrases to cover the range of topics (namely, respiratory effects, respiratory symptoms, breathing, lung, pulmonary, and respiration). The literature search was expanded by including all combination pairs of the three main search terms.

Provided below is a search strategy sample from the PubMed online database:

(1) (Wastewater treatment plant OR sewage plant) AND (respiratory effects OR respiratory Symptoms OR breathing OR lung OR pulmonary OR respiration) AND hazards.

After completing the searches and excluding duplicate studies, two of the reviewers (KM and SMY) independently screened the identified articles' titles and abstracts to select relevant articles to be included for a full review. They also reviewed citations to seek several potential and relevant articles for inclusion. In the event that there was a difference of view or opinion for the study in fulfilling the inclusion or exclusion criteria, the third and fourth reviewers (ZI and ARI) engaged to resolve those issues.

A difference in opinion occurred with regards to the suitability of selecting environmental assessment studies to be included in this review apart from human observational studies. Therefore, the reviewers decided to select only the environmental assessment studies that were conducted in sewage plants and assessed the related potential occupational respiratory hazards. Next, the articles chosen for full review were rescreened to ensure that the inclusion criteria were met. All the results were then transferred to the reference management software Endnote X7 to help the authors systematically manage the manuscripts and retrieve full-text articles. A flowchart of the literature search is shown in **Figure 1**.

Quality Assessment

The human observational and environmental assessment studies' quality was assessed using the Effective Public Health Practice Project (EPHPP) quality assessment tool for quantitative studies (see **Tables 3**, **4**) (41), which evaluates the following items: selection bias, study design, confounders, data collection methods, and withdrawal/dropout rate. Guidance for article bias ratings was taken from the EPHPP quality assessment tool dictionary. The descriptions of the mentioned items are summarized as follows: Selection bias considers to what extent study participants are likely to be representative of the target population as well as the proportion of selected individuals who agree to participate in the study. Study design considers the likelihood of bias in the allocation process for observational designs and, for experimental designs, the extent to which assessments of exposure and outcome are likely to be independent. Confounding assesses how far the essential study variables are controlled for during the data analyses and/or in the study design (by stratification or matching). Blinding examines detection and reporting bias, such as whether the researchers, persons providing the intervention, data collectors, and data analysts were aware of the research condition and/or the subjects were aware of the research question(s). Data collection methods are rated on how valid and reliable the tools for primary outcome measures, including distinctions between self-reported data; objective data retrieved by investigators, such as assessment or screening; and extracted data from medical records or vital statistics, are, and withdrawals or dropouts examines the percentage of participants remaining in the study—in other words, who managed to complete the study through the final data collection period (if applicable).

Each of these items, if applicable, was rated as having a low, moderate, or high risk of bias based on the EPHPP quality assessment tool standard guidelines. Subsequently, the overall outcomes from each domain were then translated into a global rating. The global ratings for the studies are as follows; weak-quality (in which two or more factors are rated as weak), moderate-quality (in which one aspect is rated as weak), and strong-quality (in which no characteristics are rated as weak) (42). Study assessments of quality were conducted independently by all authors (KM, SMY, ZI, and ARI). Discussions resolved any discrepancies and queries that arose during the review process.

Risk of Bias

The authors decided to assess the risk of bias pertaining to the human observational studies done at sewage plants based on three common study biases: information, selection, and confounding biases (43–45). To evaluate the information bias, potential occupational respiratory hazard exposure levels were classified into individual or ecological measurements. Ecological measurement bias might come from environmental assessment tools or monitors placed at the worksites from using job

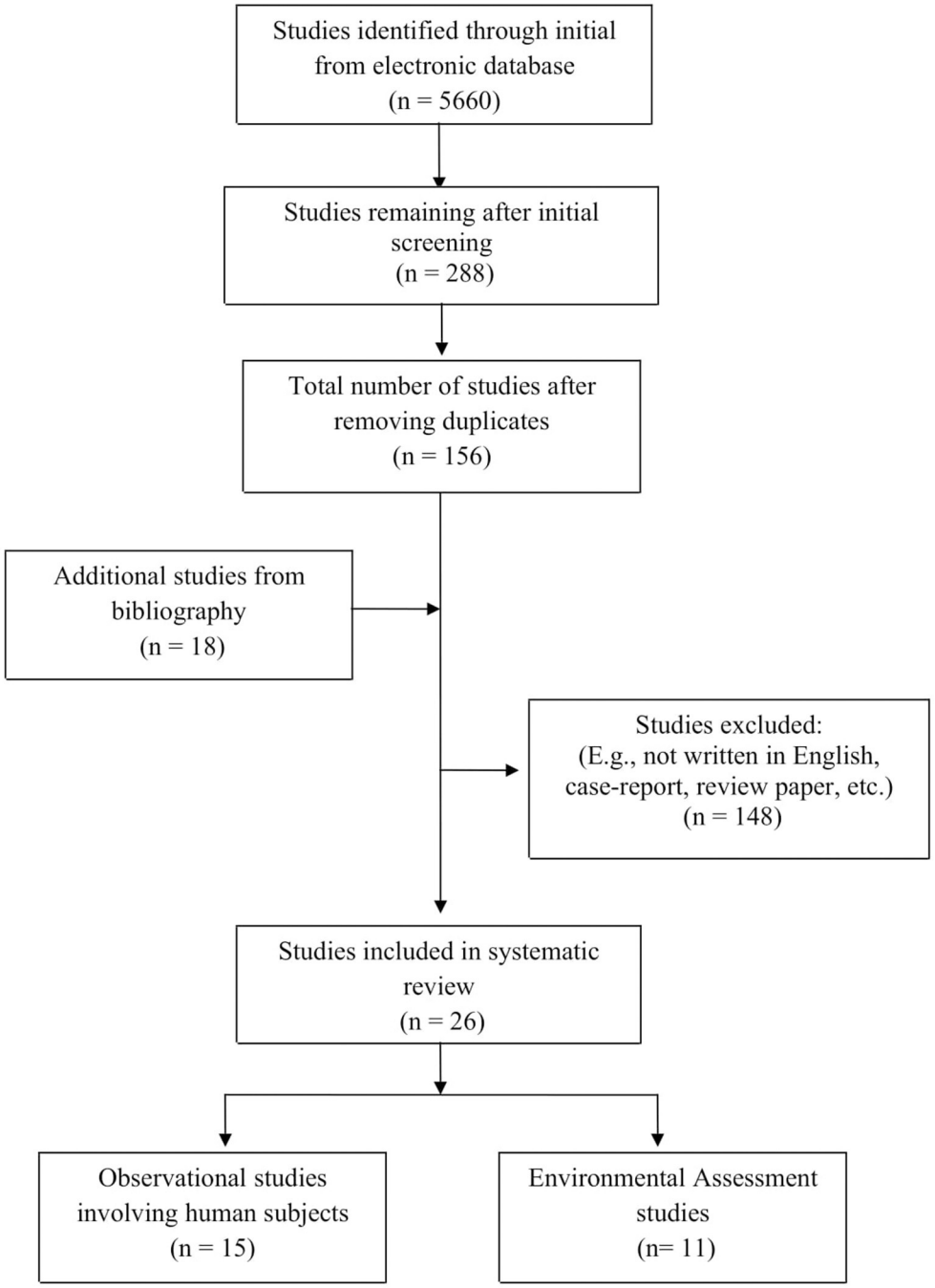

FIGURE 1 | Literature search strategy and selection process for included studies ($n = 26$).

tasks as categories of exposure, improper air-quality assessment conducted by the researchers, and selected worksites as a measure of exposure. Individual measurement bias may be present via a personal sampler measuring instrument on the sewage workers' bodies. The measurement might be distorted by contaminated work clothing, improper monitoring of workers' activities while conducting the assessment, and other sources of exposure at the sewage plants.

The confounders present in the studies were also assessed. An example of confounders would be the association between respiratory hazard exposure and respiratory health effects that might be distorted by smoking. Cigarette smoking indirectly

may contribute to the increase in particulate matter 2.5 (PM 2.5) concentration in the air and the development of respiratory illnesses (46). Thus, studies were classified by whether or not they controlled for potentially confounding variables. Last, the authors looked into the possibility of selection bias in all these studies. The selection bias can result from selecting a nonrepresentative sample of the study population. For instance, workers who are exposed to potential occupational respiratory hazards at sewage plants, such as bioaerosols, and experience respiratory symptoms may join the study more often than workers without respiratory symptoms. Studies were classified as to whether they applied a "convenience sample" or "others" for the subject selection. Subject selection was done through a convenience sampling method, such as volunteers being subjected to selection bias. This may result in misleading findings and conclusions.

RESULTS

Study Selection

Figure 1 shows a flowchart of the literature search with 5,660 research articles identified through an initial online database search. There were 18 additional studies retrieved from the bibliography search. After the screening process, we removed duplicates and ineligible items and were left with only 26 articles to be included in the systematic review.

Study Characteristics and Risk of Bias

Tables 1, **2** summarize the study characteristics of the 26 eligible studies. The tables provide information on study designs, study locations, number of participants, risk of bias, and potential respiratory hazards found in each study. **Table 1** summarizes 15 observational studies among human subjects, and **Table 2** summarizes 11 environmental assessment studies done at sewage plants.

Results of Human Observational Studies

The quality of the human observational studies included in this review was assessed with most studies (12 out of 15) having a moderate-quality rating, two rated as weak, and one rated as strong. Quality assessment results can be seen in **Table 3**. Regarding the sewage workers' exposure to potential occupational respiratory hazards found at sewage plants (see **Table 5**), most of the articles (12 out of 15) studied "endotoxin" exposure. Seven out of 15 articles studied the effects of hydrogen sulfide (H_2S) exposure among sewage workers. Next, four articles focused on inhalable dust as a potential respiratory hazard. However, two articles studied the presence of nitric oxide (NO), nitrogen dioxide (NO_2), ammonia (NH_3), sulfur dioxide (SO_2), and formaldehyde. Concerning the implemented study design, most were conducted cross-sectionally (12 out of 15), and three studies implemented a longitudinal study design.

H_2S Exposure Among Sewage Workers

Al Batony et al. (15) conducted a moderate-quality cross-sectional study among 86 subjects to determine the effects of H_2S exposure among sewage workers at a wastewater treatment plant (WWTP) in Egypt. They found the exposed workers prone to respiratory symptoms (wheezing and asthma) ($p < 0.05$) and a significant decrease in the mean value of FEV% and PEF% from the spirometry assessment ($p < 0.05$). They also found that the exposed workers had a higher mean of sulfhemoglobin percentage than nonexposed workers ($p < 0.001$). The same findings were discovered in a moderate-quality study done in 1995 by Richardson (23); the author found that the sewer workers had lower FEV1/FVC values than water treatment workers 11.0 ($p = 0.03$) and -7.8 ($p = 0.06$), respectively, after adjusting for smoking habits. These significant findings were consistent with the higher exposure of H_2S recorded among sewer workers when compared to water treatment workers. Both of these studies emphasize H_2S as the potential respiratory hazard found in sewage plants and significantly affecting the workers' respiratory health.

H_2S and Other Chemical Air Pollutant Exposure Among Sewage Workers

We found five articles studying more than one substance in addition to H_2S. One moderate-quality study looked into H_2S and other chemicals, such as NO_2, SO_2, NH_3, and formaldehyde (25). The study was conducted in the old and new STPs in Cairo city and measured the effects of those exposures on workers' respiratory health. They established significantly lower mean values of FVC% of predicted exposure to all studied respiratory hazards among both groups of STP workers than controls. The aeration tank workers at the new plant and screening tank workers at the old plant recorded the lowest mean values of FVC% predicted 57.9 $\pm$ 10.7 and 54.0 $\pm$ 13.9, respectively. Different STPs used different techniques and processes, and this may produce various sources of hazards.

H_2S and Endotoxin Exposure Among Sewage Workers

Meanwhile, the other four articles studied the effect of H_2S and endotoxin exposure among sewage workers. The latest study was conducted by Heldal et al. (20); this is the only strong-quality study found in this review. It is a longitudinal study done among 148 sewage workers in Sweden. In this study, the authors planned to take samples over a year, including four seasons. The authors found that only 9% of all H_2S samplings recorded a peak above 10 ppm in which the threshold limit value (TLV) is 1 ppm, and the highest exposure was among sewage workers who do the job of collecting sewage from a cesspool (273 ppm). Next, there was a significant negative association between exposure of endotoxins and FEV1% value among sewer net workers, which means that the higher endotoxin exposure among workers produced a lower FEV1% value.

Additionally, there was a significantly lower FEV1 and FVC% among sewage workers than referents after adjusting for age, smoking, and BMI. The endotoxin and H_2S exposure among the subjects were confirmed to be associated with seasonal variation and working operation. A weak-quality study done among sewage workers in Sweden reported that endotoxin exposure might contribute to developing respiratory symptoms among the subjects (28). However, this study's findings were questionable

TABLE 1 | Human assessment (observational studies) done at the sewage industry included in the systematic review.

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Al Batanony et al. (15)	Cross-sectional	Sewage workers at Berket Al-Sabih WWTP, Egypt and Workers at Departments of faculty of Commerce, Menoufiya University, Egypt	86 subjects: 43 exposed and 43 non exposed	Interview, Spirometry examination, 12-lead Electrocardiogram (ECG), Quantitative sandwich enzyme linked immunosorbent assay (ELISA), Polymerase chain reaction (PCR)	Exposed workers had a significant decrease in the mean value of FEV % and PEF %. Exposed workers had significantly higher mean sulf-heamoglobin % as compared to non exposed workers.	I	Controlled (Age and smoking habit)	O	Hydrogen Sulfide	Moderate
Cyprowski et al. (16)	Cross-sectional	Combined STP in Central Poland	78 STP workers	Endotoxin assessment via personal aerosols samplers, Spirometry examination	Sewage sludge treatment workers (SSTW) recorded the highest exposure level to Endotoxin (89.5 EU/m^3) and inhalable dust (0.24 mg/m^3). There was a weak positive correlation between the level of inhalable dust and endotoxin concentration ($p = 0.003$, $r = 0.33$). Low levels of endotoxin exposure among workers contributed to a significant impact on declining in FEV1 ($p = 0.044$)	I	Controlled (Inhalable dust exposure and smoking habit)	O	Inhalable dust, Endotoxin	Moderate
Douwes et al. (17)	Cross-sectional	Dutch STP workers	151 STP workers (only 147 returned the questionnaire)	Self-reported health questionnaire, Personal inhalable dust and endotoxin samplers	The upper and lower respiratory symptoms were significantly higher in respondents working 5–10 years ($p < 0.001$) Flu-like symptoms were significantly associated with exposure to sewage (OR = 5.0). No difference in endotoxin and inhalable dust exposure were observed between both plants and between the different seasons. Relatively low endotoxin and inhalable dust exposure among the subjects recorded with a geometric mean of (9.5 EU/m^3 and 0.3 mg/m^3) respectively.	I	Controlled (Age and smoking)	C	Endotoxin, Inhalable dust (NS)	Weak

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Heldal et al. (18)	Cross-sectional	Eight municipal sewage plants workers in Norway	80 subjects: 36 unexposed and 44 exposed	Self-administered questionnaire, Spirometry examination, Personal Air Sampler Chemi-luminescence Analyzer to measure nitric oxide, Acoustic rhinometry to measure the cross-sectional area and volume of the nasal passage, C-reactive protein blood investigation	Sewage workers handling dry sludge had a higher prevalence of respiratory symptoms and significantly lower FEV1/FVC ratio as compared to non-dry sludge workers ($p < 0.001$). Endotoxin and inhalable dust exposure were significantly higher among sludge treatment workers as compared to plant workers who not involved with sludge treatment ($p < 0.05$). Nitric monoxide exposure among sewage workers was not significant as compared to controls	I	Controlled (Age and smoking)	O	Inhalable dust, Endotoxin, Nitric monoxide (NS)	Moderate
Heldal et al. (19)	Cross-sectional	Eight municipal sewage plants workers in Norway	82 subjects: 44 cases and 38 controls	Self-administered questionnaire, Personal Air Sampler, Blood samples for CC16, SP-A, and SP-D to measure pneumoproteins	Positive correlations between endotoxin and dust concentrations ($rp = 0.47$, $p < 0.01$) and between endotoxin and bacteria concentrations ($rp = 0.37$, $p < 0.05$). Exposed subjects had significantly lower mean concentration of CC16 in serum as compared to the referents ($p = 0.008$)	I	Controlled (Age, sex, atopy, and smoking)	O	Inhalable dust, Endotoxin, Bacteria	Moderate

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Heldal et al. (20)	Longitudinal	Sewage workers from four STPs in small rural communities (Steinkjer, Støren, Klæbu, Selbu)	148 subjects: 121 cases and 27 referents (control)	Self-administered questionnaire, Spirometry examination, Personal Air Sampler, Blood samples (to measure ICAM-1, CRP, MIP-1alpha, interluekin-8, CC 16, and surfactant protein D (SP-D)	The highest exposure level of endotoxin was recorded at the sewer net system (342 EU/m^3) and the lowest exposure level at grease handling with median (13 EU/m^3). The exposure was significantly associated with the working operation ($p < 0.05$) and season ($p < 0.05$). Only 9% of all H_2S recording shown a peak at above (10 p.p.m). The job with the most excessive exposure to H_2S was collecting sewage from cesspools (273 p.p.m.). Sewage plant and sewer workers had a significantly higher prevalence of work-related airway symptoms as compared to referents (33 and 11%, respectively). Noted a significant lower in FEV1% and FVC% among sewage plant and sewer workers as compared to referents. No significant difference in spirometry parameters among sewage workers between the studied sewage plants. There was negative association between ICAM-1 and exposure to H_2S among sewer net workers ($\beta = -52.6$, $r^2 = 0.07$, $p < 0.05$). There was negative association between FEV1 and Endotoxin exposure among sewer net workers ($\beta = -0.22$, $r^2 = 0.18$, $p < 0.05$).	I	Controlled (Age, smoking, body mass index, and atopy)	O	Endotoxin, Hydrogen Sulfide	Strong

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Lee et al. (21)	Cross-sectional	4 Wastewater treatment plant (WWTP), Iowa, United States and 21 water treatment plant (WTP), Iowa, Unites States	147 Subjects: 93 WWTP workers and 54 WTP workers	Self-administered questionnaire, Spirometry examination, Personal Air Sampler of hydrogen sulfide and endotoxin	WWTP workers had a significantly higher prevalence of respiratory symptoms as compared to WTP workers (OR 2.7, 95% CI; 1.1–72.7). The majority of H_2S samples (95.2%) were less than the threshold limit value (TLV) of 1 p.p.m. 64.5% of the endotoxin sample concentrations exceeded 50 EU/m^3. WWTP workers suffered from respiratory symptoms known to be associated with hydrogen sulfide exposure. There was no significant association between endotoxin exposure and the development of respiratory symptoms among the WWTP and WTP workers.	I	Controlled (Smoking, use of respirator, and allergy)	O	Endotoxin, Hydrogen Sulfide	Moderate
Melbostad et al. (22)	Longitudi-nal	15 municipal STP in eastern Norway	24 sewage workers	Health symptoms reporting system, Personal sampling pump	No significant correlation between exposure of endotoxin and bacteria ($r^2 = 0.00$). There was a significant association between exposure to rode-shaped bacteria and the presence of tiredness ($p < 0.05$) and headache symptoms ($p < 0.05$). There was no significant relationship between symptoms and exposure to hydrogen sulfide and endotoxin during a work shift.	I	Did Not Control	O	Bacteria Endotoxin (NS), Hydrogen Sulfide (NS)	Moderate

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Richardson (23)	Cross-sectional	Sewer and water treatment workers in Durham and Winston-Salem, North Carolina	223 Subjects: 107 sewer workers and 116 water treatment workers	Self-administered questionnaire, Spirometry examination, Personal Air Sampler of hydrogen sulfide and endotoxin. Job titles were used to categorize the presumed levelof occupational H,S exposure.	Sewer workers appeared to have lower lung function values than water treatment workers. FEV,/FVC values in the smoker and non-smoker sewer workers were statistically significantly lower compared to water treatment workers 11.0 ($p = 0.03$) and −7.8 ($p = 0.06$), respectively. Workers classified as high H_2S exposure consistently had the lowest observed FEV1/FVC values.	I	Controlled (Age, height, race, and Smoking)	O	Hydrogen Sulfide	Moderate
Rylander (24)	Cross-sectional	Sewage workers in eight sewage treatment plants in four municipalities in the south of Sweden	69 subjects: 34 sewage workers (cases) and 35 controls	Interviewed administer questionnaire, Spirometry examination, Stationary air sampler (Air sampling filters)	The vicinity of sludge handling and during cleaning were recorded as the highest amount of airborne endotoxin range 2–32 170 ng/m^3). However, the amount of endotoxin presence at sewage treatment plants was not exceeded the normal background value. Sewage workers had a higher prevalence of airway symptoms as compared to controls after adjusted by smoking status. The Methacholine induced decrease in FEV1 was significantly higher among sewage workers compared to controls −6.2 (5.8) and −4.8 (3.9), respectively	IE	Controlled (Smoking and subjects who had past exposure in dusty industry)	O	Endotoxin	Moderate

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Saad et al. (25)	Cross-sectional	Old STP located at the South-west of Cairo city, New STP located at the South-west of Cairo city	61 newSTP workers and 46 old STP workers and compared with 40 non-exposed controls	Self-administered questionnaire, Spirometry examination, Stationary air sampler using calibrated vacuum pump, dry gas meter, and large glass bubblers	Sewage workers working at the new plant had a significantly higher prevalence of developing acute bronchitis ($p < 0.05$) compared to controls. Both plants (new and old) had significantly lower mean FVC% of predicted sewage workers as compared to controls. Old plants sewage workers had significantly lower FEV1% of predicted and the FEV1/FVC% value compared to controls. The highest concentration of H_2S and NH_3 were found at screening tanks of new plants. While the aeration tanks of the old plant recorded the highest concentration of NO_2. Exposure to hazardous chemical pollutions (H_2S, NH_3, NO_2, SO_2, and HCHO) among the sewage workers caused a significant effect on pulmonary function.	IE	Controlled (Smoking)	O	Hydrogen sulfide, Nitrogen dioxide, Ammonia, Sulfur dioxide, Formalde-hyde	Moderate
Smit et al. (26)	Longitudi-nal	Twenty seven Dutch Water Boards workers	468 subjects: 97 office workers, 371 operators and maintenance workers	Self-administered adapted version of questionnaire, Personal endotoxin exposure sampler	Personal 8 h endotoxin exposure was low with a geometric mean of (27 EU/m^3). Wastewater workers had a higher prevalence of having cough symptoms compared to the general population ($p < 0.001$). Wastewater workers who were exposed to the highest level of endotoxin (>200 EU/m^3) had a higher prevalence ratio in having flu-like and upper respiratory symptoms [PR: 2.02 (95% CI: 0.83–4.88)] and [PR: 1.79 (95% CI: 0.84–3.84)].	I	Controlled (Age, gender, and smoking habits)	C	Endotoxin	Moderate

(Continued)

TABLE 1 | Continued

References	Study design	Study location/subjects	Number of subjects	Assessments	Outcomes	Risk of bias			Potential hazards	Global ratings
						Information	Confounding	Selection		
Tabrizi et al. (30)	Cross-sectional	Wastewater and garbage workers in Canton, Zurich	778 Subjects: 395 controls and383 cases (316 wastewater workers 67 garbage workers)	Self-administered questionnaire, Spirometry examination, Personal Air Sampler of Endotoxin, ELISA	The mean exposure levels to endotoxins in both plants were below 100 EU/m^3. Special tasks of wastewater workers caused higher endotoxin exposure with a mean of 98.6 EU/m^3 (1.4–497). There was no significant difference in the level of FEV1/FVC between the 3 groups ($p > 0.1$). No clinically relevant correlation between spirometry results and SP-D concentrations appeared.	I	Controlled (Age, gender, previous or current work as a farmer, and smoking habits)	O	Endotoxin	Moderate
Thorn (28)	Cross-sectional	five municipal STP in western Sweden	114 Subjects: 59 operatives workers (cases) and 55 controls	Self-administered questionnaire, Spirometry examination, Personal and Stationary Air Samplers, ELISA, PCR, Nasal Lavage	The prevalence of presence respiratory symptoms among cases was higher than cases and most likely due to endotoxin exposure. No significant difference in pulmonary function values between the cases and controls. Most of the H_2S reading was low (<1 p.p.m). the highest recorded at 6 p.p.m	*IE*	Did not control	O	Endotoxin, Hydrogen Sulfide	Weak
Tschopp et al. (29)	Cross-sectional	Wastewater and garbage workers in Canton, Zurich	603 Subjects: 304 controls, 299 cases (247 wastewater and 52 garbage workers)	Interviewed and Self-administered questionnaire, Spirometry examination, Personal and stationary Air Sampler of Endotoxin, ELISA	The highest peak exposure of endotoxin up to 500 EU/m^3 was identified among wastewater workers. No significant association between exposure to endotoxin and respiratory symptoms development, spirometry value, and specific protein concentration throughout the study between the subgroups. The effect of occupational exposure toward endotoxin was not significant	I	Controlled (Obesity, previous or current work as a farmer, and smoking habits)	O	Endotoxin	Moderate

E, Ecological; I, Individual; C, Convenience; O, Others; NS, Not Significance.

OR, Odds ratio; PR, Prevalence ratio; H_2S, Hydrogen sulfide; NO_2, Nitrogen dioxide; NH_3, Ammonia; SO_2, Sulfur dioxide; HCHO, Formaldehyde; PM 2.5, Particulate matter 2.5; VOC, Volatile organic compound; FEV1, Forced expiratory volume in 1s; FVC, Forced vital capacity; PEF%, Peak expiratory flow percent of predicted value; FEV%, Forced expiratory volume percent of predicted value; WWTP, Wastewater treatment plant; STP, Sewage treatment plant; SSTW, Sewage sludge treatment workers; COPD, Chronic obstructive pulmonary disease; ELISA, Enzyme linked immunosorbent assay; PCR, Polymerase chain reaction; SP-D, Surfactant protein D; SP-A, Surfactant protein D; CRP, C-reactive protein; ICAM, Intercellular adhesion molecule; MIP, Int macrophage inflammatory protein; CC16, Club cell protein 16.

TABLE 2 | Environmental exposure assessment studies done at the sewage industry included in the systematic review.

References	Study location	Number of samples	Assessments	Outcomes	Potential hazards	Global ratings
Austigard et al. (30)	Two areas:Two WWTP in the cities and three WWTP in the rural area, middle of Norway	93 sample measurements,149 sewage workers were selected and divided into four group: (i) Big plants (ii) Pumps station (iii) Sewer network (iv) Collecting sewage from cesspools	Personal air sampling for H_2S	8 out of 93 samples (9%) of H_2S measurements had recorded peaks above 10 p.p.m. Workers working at collecting sewage from cesspool station had the highest level of exposure to H2S. The determinants that could affect the H2S index were job type, season, plant location, and degree of flushing.	H_2S	Strong
Carducci et al. (31)	WWTP in Italy	Numbers of samples not mentioned. Four locations of study: (i) Sewage effluent (ii) Biological oxidation tank (iii) Sludge treatment (iv) Side entrance manhole	Secondary data, Stationary air sampling for bioaerosol using impactor sampler loaded with Rodac plates, QMRA	Workers who worked in the sewage effluent and biological oxidation tank had a higher probability of illness in relation to the exposure of the bioaerosols with very good reliability ($r^2 = 0.92$). There were estimated higher quantitative microbial average risk exposure in sewage influent and biological oxidation tanks (15.64 and 12.73% for an exposure of 3 min). Human adenovirus concentration is a predominant factor in the estimated risk.	Bioaerosol (Human Adenovirus)	Moderate
Cyprowoski et al. (32)	WWTP in Poland	Six sampling points of both sewage and sludge station, at 10 different workplaces in WWTP	Stationary air sampling using 6-stage Andersen impactor, Petri plates containing Schaedler agar. Samples collected at single repetition in July 2014 and February 2015.	The average concentration of anaerobic bacteria in the sewage samples was 5.49 × 104 CFU/m^3 (GSD = 85.4) and in sludge 1.42 × 106 CFU/g (GSD = 5.1). Winter at the bar screens recorded the highest bacterial contamination (4.06 × 103 CFU/m^3). 16 bacterial species were determined, from which the predominant strains belonged to 5 genera which were; *Actinomyces, Bifidobacterium, Clostridium, Propionibacterium,* and *Peptostreptococcus* genera. Mechanical treatment processes caused a substantial emission of anaerobic bacteria into the air. No significant difference in bacterial biota in the air between sewage and sludge station.	Bioaerosol (Airborne Anaerobic Bacteria)	Strong

(Continued)

TABLE 2 | Continued

References	Study location	Number of samples	Assessments	Outcomes	Potential hazards	Global ratings
Gotkowska et al. (33)	Domestic WWTP, Ostro'da in the north-east of Poland	288 air samples were taken: Contorls = 24, Mechanical treatment = 96, Biological treatment = 72, and surroundings = 96 12 sampling sites: (i) 1 control site (ii) 7 sites located in the WWTP (grate chamber, grit chamber, retention chamber, preliminary settling tank, pre-denitrification tank, nitrification, and denitrification tanks, secondary sedimentation tank (iii) 4 sites located outside the WWTP (at the fence of the plant, and 50, 100, and 200 min from the fence)	Stationary air sampling via impact sampler surface air system MAS-100 Eco Merck with 400 holes, Petri plates containing agar. Air sampling taken in two annual cycles (in 2005 and 2006) and in four different seasons.	Overall, 25 species of microorganisms were identified. Higher numbers of HPC bacteria in air samples were observed in summer, fungi in autumn. The mechanical sewage treatment produced significant emission of microorganisms to the air (the grate chamber, the grit chamber, the preliminary settling tank). While biological sewage treatment equipped with a fine bubble aeration system produced only a small amount of bioaerosols.	Bioaerosol (Airborne Bacteria)	Strong
Oppliger et al. (34)	11WWTP in Canton of Zurich, Switzerland.	22 air samples from 11 sewage treatment plant (indoor and outdoor)	Personal air sampling using polycarbonate filters, Stationary air sampling, Agar plates. Air sampling taken in two season which were winter and summer. Four hours sampling in two reading per day.	The fungi concentration was significantly higher in summer as compared to the winter season (2,331 ± 858 vs. 329 ± 95 CFU/m^3). Particle grids for incoming water in the enclosed areas had a significantly higher bacteria concentration as compared to the unenclosed areas near the aeration basins in both winter and summer seasons. All bioaerosols were frequently above the recommended values of occupational exposure. The sewage workers who needed to conduct special tasks such as cleaning tanks were exposed to a very high levels of endotoxins (up to 500 EU/m^3) compared to routine work. The most predominant species of bacteria found were from Pseudomonadaceae and the Enterobacteriaceae family.	Bioaerosols (Airborne Bacteria)	Moderate
Shiota et al. (35)	Sewage sludge incinerators (SSI) in Japan	5 sampling points: (i) 2 dry Electrostatic precipitators (EP) (ii) 1 wet EP (iii) 1 bag filter (iv) 1 ceramic filter	Andersen stack samplers to measure particulate matter	The average PM 2.5 concentration was 0.00014–4.8 mg/Nm^3. Annually, an estimated about 0.96–8.9 tons of PM 2.5 was emitted into the air. The highest contribution of PM 2.5 emission was at dry EP (77–99% of total emission).	PM 2.5	Moderate

(Continued)

TABLE 2 | Continued

References	Study location	Number of samples	Assessments	Outcomes	Potential hazards	Global ratings
Spaan et al. (36)	43 Dutch sewage treatment plants	647 air samples were taken: (i) 470 full shift personal (ii) 123 tasked-based (iii) 54 stationary measurements	3 occupational exposure assessments methods applied: Personal, Stationary, Task-Based	There was moderate to low endotoxin exposure among the sewage treatment plant workers. The highest exposure recorded was through task-based method and followed by stationary and the least was personal sampling with an overall geometric mean of 64, 33, and 27 EU/m^3, respectively. No significant determinants of day to day exposure variability within and between the workers. The highest endotoxin levels recorded via stationary measurements were found in the front end of the process has, whereas the highest dust concentrations were found during sludge dewatering. The highest amounts of total bacteria counts found in the sludge dewatering system were gram-positive bacteria and fungi levels were higher as compared to gram-negative bacteria levels.	Bioaerosol (Endotoxin and Bacteria)	Moderate
Upadhyay et al. (37)	WWTPs in the Phoenix (AZ) metropolitan area	24 air samples were taken 2 main locations: (i) Covered area basin (CAB) (ii) Open area basin (OAB)	Field sampling methods: PM sampling system, annular denuders to measure ammonia concentration, ChemVol impactor to measure organic trace. Air sampling taken within 1 year duration of interval.	The concentrations of PM 2.5, PM10, and NH3 at the aeration basins were similar and within urban ranges. PM 2.5concentration at the CAB basin was the highest compared to other samples. NH3 concentrations were highest at the CAB facility (13 to 72 mg/m^3) above the aeration basin. However, there was no significant difference between NH3 concentration at both location CAB and OAB.	PM 10, PM 2.5, Ammonia	Strong
Yang et al. (38)	WWTP at Harbin City in northern China	Numbers of samples not mentioned	Stationary air and water sampling for VOCs. Four sampling seasons were monitored.	Three aromatic hydrocarbons, notably benzene, were more readily released from the wastewater into the atmosphere. The primary clarifier of the WWTP had the highest VOC concentrations during summer. VOC especially polyaromatic hydrocarbon expected had long-distance traveling to the surrounding area as was observed at locations far away from the WWTP.	VOC	Strong

(Continued)

TABLE 2 | Continued

References	Study location	Number of samples	Assessments	Outcomes	Potential hazards	Global ratings
Yang et al. (39)	WWTP located in Beijing, China.	7 sites: (i) Coarse screen (CS) (ii) Aerated grit chamber (AGC) (iii) Primary settling tank (PST) (iv) Anaerobic tank (AnT) (v) Aeration tank (AeT) (vi) Secondary settling tank (SST) (vii) Sludge dewatering house (SDH)	Stationary air sampling for bioaerosol, Total suspended particles (TSP) sampling, Nutrient agar to measure airborne bacteria, Ion Chromatography System to measure anions concentration. Three sampling for each season; winter spring and summer.	There were positive correlations between sites and bacterial concentrations were observed in winter, spring, and summer (ANOVA 1, $p < 0.001$). The highest emission level of airborne bacteria recorded at treatment stages of CS, AGC, PST, AnT, and AeT ranged from 257 to 4,878 CFU/m^3. The concentration of airborne bacteria was significantly lower at the external sites of WWTP as compared to internal sites of WWTP ($p < 0.001$). Assume to be due to wind dilution and dispersion effect. The main anions were detected; chloride, nitric oxide, and sulfur dioxide. Inhalation risk magnitude is higher among the WWTP workers as compared to skin contact risk especially at worksites with high levels of airborne bacteria, such as AGC, CS, and AeT. Inhalation risks of airborne bacteria in summer were higher than those in the other two seasons.	Bioaerosol (Airborne bacteria), NO, SO_2, Cl	Srong
Prazmo et al. (40)	Sewage treatment plant located in eastern Poland	12 sites: (i) 9 at Sewage treatment Plant (ii) 2 at City pump station (iii) 1 sewer duct	Stationary air sampling for airborne endotoxin Agar media. Five double air samples taken from each site.	The sampling site was a significant factor in determining quantities of airborne microorganisms ($p < 0.001$). The initial phase of STP processes which were clearing, primary sedimentation, aeration had 2–3 times higher airborne microorganism loads compared to those at final phase STP processes which were secondary sedimentation and sludge dewatering. The majority of bacteria found in eight sites were corynebacteria. Three-quarter of the samples shown positive fungi aerosols. The airborne endotoxin concentration in the plant was low and within the range of 0.104–5.2 ng/m^3. The concentrations of microorganisms and endotoxin were not significantly correlated ($p > 0.05$).	Bioaerosol (Airborne endotoxin), Bacteria, Fungi	Weak

H_2S, Hydrogen sulfide; NO, Nitrogen oxide; SO_2, Sulfur dioxide; Cl, Chloride; PM 2.5, Particulate matter 2.5; VOC, Volatile organic compound; WWTP, Wastewater treatment plant; STP, Sewage treatment plant; QMRA, Quantitative microbial risk assessment; HPC, Heterotrophic bacteria; CFU, Colony forming unit; GSD, Geometric standard deviation.

TABLE 3 | Quality assessment results against the effective public health practice project quality assessment tool for human observational studies.

References	Selection bias	Design	Confounders	Blinding	Data collection method	Withdrawals/dropouts	Global ratings
Al Batanony et al. (15)	Moderate	Weak	Moderate	Moderate	Strong	Strong	Moderate
Cyprowski et al. (16)	Moderate	Weak	Moderate	Moderate	Strong	Strong	Moderate
Douwes et al. (17)	Moderate	Weak	Weak	Moderate	Strong	Strong	Weak
Heldal et al. (18)	Strong	Weak	Moderate	Strong	Strong	Strong	Moderate
Heldal et al. (19)	Strong	Weak	Strong	Strong	Strong	Strong	Moderate
Heldal et al. (20)	Strong	Moderate	Strong	Moderate	Strong	Strong	Strong
Lee et al. (21)	Strong	Weak	Strong	Strong	Strong	Moderate	Moderate
Melbostad et al. (22)	Moderate	Moderate	Weak	Strong	Strong	Strong	Moderate
Richardson (23)	Moderate	Weak	Moderate	Strong	Moderate	Moderate	Moderate
Rylander (24)	Strong	Weak	Strong	Strong	Strong	Strong	Moderate
Saad et al. (25)	Strong	Weak	Moderate	Strong	Strong	Strong	Moderate
Smit et al. (26)	Moderate	Weak	Strong	Strong	Strong	Strong	Moderate
Tabrizi et al. (27)	Moderate	Weak	Strong	Moderate	Strong	Strong	Moderate
Thorn (28)	Strong	Weak	Weak	Strong	Moderate	Strong	Weak
Tschopp et al. (29)	Moderate	Weak	Strong	Moderate	Strong	Strong	Moderate

as this study did not control for any confounders during the selection of subjects or in the data analysis.

On the other hand, two moderate-quality studies done by Lee et al. (21) and Melbostad et al. (22) did not include or discuss seasonal variation effects. Lee et al. (21) found most H_2S samples collected in the study were less than TLV of 1 ppm. However, it was found that a higher prevalence of WWTP workers had respiratory symptoms compared with WTP workers, and this was associated with H_2S exposure. In comparison, exposure to endotoxins was not found to be related to the development of respiratory symptoms in both worker groups. However, more than half of the study's air samples exceeded endotoxin concentration (>50 Eu/m^3). Paradoxically, there was no association between H_2S and endotoxin exposure among 24 sewage workers during the work shift in eastern Norway (22). However, the authors found a significant association between exposure to bioaerosol (bacteria) and the presence of tiredness symptoms ($p < 0.05$).

Endotoxin Exposure Among Sewage Workers

Next, four moderate-quality articles looked into endotoxin exposure and its effect on sewage workers' respiratory health. Two out of the four studies show that there is a significant association between exposure to endotoxins and respiratory health among sewage workers [i.e., development of respiratory symptoms (26) and deterioration of pulmonary function (24)]. Rylander et al. (24) conducted a study among 69 sewage workers in south Sweden. They establish endotoxins at the sewage plants, and the highest concentration recorded at the sludge handling and cleaning section ranged from 3.8 to 32 170 ng/m^3. The endotoxins were confirmed to cause airway inflammation.

Another study in the Netherlands at 27 sewage plants reveals a low geometric mean of endotoxin exposure (27 Eu/m^3) found in the study. Wastewater workers exposed to a higher level of endotoxins (>200 Eu/m^3) had a higher prevalence ratio in developing flu-like and respiratory symptoms. In contrast, another two articles prove no association between the level of endotoxin exposure and the presence of adverse respiratory health effects (27, 29). Both of these studies were conducted among wastewater and garbage workers in Zurich. The authors from both studies found that endotoxin exposure in both plants was low (<100 Eu/mg^3). Also, endotoxin exposure in both groups of workers' spirometry values was not significant throughout the subgroups after adjusted for smoking and obesity.

Inhalable Dust Exposure Among Sewage Workers

Inhalable dust is also a potential respiratory hazard found at sewage plants and is shown to be associated with the development of respiratory symptoms and decreased lung function. These facts are proven in two moderate-quality studies by Heldal et al. (18, 19). The authors conducted a cross-sectional study at eight municipal plants in Norway. They established there was endotoxin and inhalable dust exposure among the workers. The exposures were significantly higher among sludge treatment workers as compared with workers not involved in sludge treatment. In addition, sludge workers tend to have a higher prevalence of developing respiratory symptoms and a significant reduction in spirometry values, especially the FEV1/FVC ratio ($p < 0.001$). There was also a significant positive correlation between endotoxin and dust concentration at the sewage plant ($rp = 0.47, p < 0.01$).

However, the other two studies show contradictory findings on the presence of inhalable dust exposure and its effect on sewage workers' respiratory health. First Cyprowski et al. (16) find a weak positive correlation between endotoxin and inhalable dust ($r = 0.33, p = 0.003$). The inhalable dust exposure among the workers was low and below the exposure limit. It is shown that the FEV1 significantly declined among the workers who had the highest endotoxin exposure, but it was independent of inhalable dust and smoking habits. Second a weak-quality study conducted among 151 STP workers shows no significant difference in exposure of endotoxins and inhalable dust throughout the four

TABLE 4 | Quality assessment results against the effective public health practice project quality assessment tool for environmental assessment studies.

References	Selection bias	Design	Confounders	Blinding	Data collection method	Withdrawals/Dropouts	Global ratings
Austigard et al. (30)	Strong	Moderate	Strong	Moderate	Strong	Strong	Strong
Carducci et al. (31)	Moderate	Moderate	Weak	Moderate	Moderate	Strong	Moderate
Cyprowoski et al. (32)	Strong	Moderate	Moderate	Moderate	Strong	Strong	Strong
Gotkowska et al. (33)	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Strong
Oppliger et al. (34)	Strong	Moderate	Weak	Moderate	Strong	Strong	Moderate
Shiota et al. (35)	Strong	Moderate	Weak	Moderate	Strong	Strong	Moderate
Spaan et al. (36)	Strong	Moderate	Weak	Strong	Strong	Strong	Moderate
Upadhyay et al. (37)	Moderate	Moderate	Moderate	Moderate	Moderate	Strong	Strong
Yang et al. (38)	Strong	Moderate	Moderate	Strong	Strong	Strong	Strong
Yang et al. (39)	Strong	Moderate	Moderate	Moderate	Strong	Strong	Strong
Prazmo et al. (40)	Moderate	Weak	Weak	Moderate	Strong	Strong	Weak

seasons with a geometric mean of 9.5 Eu/m^3 and 0.3 mg/m^3, respectively (17). The endotoxin and dust exposure among the workers were unable to explain the development of flu-like symptoms. It could be affected by other associated factors as well. However, this study was classified as weak quality due to the weak study design and was not properly controlled by the confounders.

Results of Environmental Assessment Studies

The quality of the environmental assessment studies included in this systematic review were assessed. Most studies (6 out of 11) have a strong-quality rating; four are rated as moderate, and one rated as weak (see **Table 4**). Regarding the method of air sampling assessment, most of the articles (7 out of 11) utilized stationary air sampling to determine and measure the potential hazard concentration at the sewage plants. Only one study used an individual or personal air sampling technique. In contrast, two studies utilized both personal and stationary air sampling techniques. On top of that, task-based air sampling and personal and stationary sampling were used in a study done by Spaan et al. (36).

The majority of the articles (5 out of 11) measured bioaerosol or endotoxin concentration present at sewage plants (see **Table 5**). These include six articles that measured bacteria concentration, and the other article studied virus concentration at sewage plants. Next, there were two articles reviewed on the presence of PM 2.5, one article on H_2S, one article on volatile organic compounds (VOC), and two articles on several chemicals (e.g., NO, chloride, NH_3, and SO_2) concentration at sewage treatment plants.

TABLE 5 | Summary of occupational respiratory hazards studies included in the systematic review.

Studied potential occupational hazards	Articles references
Human observational studies	
(1) Endotoxin	(16–22, 24, 26–29)
(2) Hydrogen sulfide	(15, 20–23, 25, 28)
(3) Inhalable dust	(16–19)
(4) Other chemicals Nitric monoxide (NO), Nitric dioxide (NO_2), Ammonia (NH_3), Sulfur dioxide (SO_2), Formaldehyde	(18, 25)
Environmental assessment studies	
(1) Bioaerosols or endotoxin	(31–34, 36, 39, 40)
(2) Particulate matter	(35, 37)
(3) Hydrogen sulfide	(30)
(4) volatile organic compound	(38)
(5) Other chemicals Nitric monoxide (NO), Chloride (Cl), Sulfur dioxide (SO_2), Ammonia (NH_3)	(37, 39)

H_2S Concentration in the Air

A strong-quality study was conducted by Austigard et al. (30) at 56 WWTPs in the middle of Norway. The authors collected 93 personal air samples over 1 year to measure the exposure of H_2S among the sewage workers and determine the effect of seasonal variation on the exposure. They found only 1 in 10 (9%) of H_2S measurements recorded were above TLV level (>10 ppm). In this study, the authors calculated the H_2S index used to evaluate the exposure and its relation to health effects. The workers who did the job of collecting sewage at the cesspool obtained the highest H_2S index. Subsequently, the study suggests that the exposure of H_2S among sewage workers could be affected by several determinants: job type, seasonal variation, location of the plant, and degree of flushing.

PM 2.5 Concentration in the Air

Two articles measured PM 2.5 emission at sewage plants. The latest study was conducted by Shiota et al. (35) in 2015. Anderson stack filters were utilized and located at five sewage sludge incinerators in Japan to measure PM 2.5 emission mass concentration. It was found that the PM 2.5 emission was low and close to the environmental standard (35 μg/m^3 daily). The SSI using the dry electrostatic precipitator method recorded the

highest contribution emission of PM 2.5. Next, 24 stationary air samples were taken from a WWTP study in the United States (37). In this strong-quality study, it was found that the emission of PM 2.5, PM 10, and NH_3 at both types of aeration basin (open and closed system) were within turban range. There were no significant differences found in the emission of those three studied substances in the air between open and closed aeration basins.

Bioaerosols or Endotoxin Concentration in the Air

It appears that the studies done so far concerning environmental air assessment at sewage plants are focused on bioaerosols and endotoxins, which include bacteria, viruses, and fungi. One moderate-quality study was conducted at a WWTP in Italy using a stationary air sampling method to quantify the quantitative microbial risk assessment (QMRA) for the human adenovirus (HAdv) among sewage workers (31). The QMRA is useful in assessing health risks at the individual level. The authors found that sewage workers who worked at sewage influent and biological oxidation tanks had a higher risk of HAdv exposure compared with sludge treatment and side entrance manholes with good reliability results ($r^2 = 0.92$). In addition, sensitivity analysis was conducted and HAdv concentration was found to be a predominant factor to be included in the QMRA.

Regarding the airborne bacteria concentration at sewage plants, a strong-quality study conducted by Cyprowoski et al. (32) tried to assess the exposure to anaerobic bacteria released into the air at the WWTP in Poland. They collected 12 samples for 6 sampling points via stationary air samplers in a year. They found that the anaerobic bacteria widely presented in the air at WWTP workplaces and mechanical treatment processes caused a significant release of anaerobic bacteria emission into the air ($p < 0.05$). Next, there were 16 bacterial species identified, but there were no significant differences in the bacteria's microbiota across the samples taken. There was also no difference in anaerobic bacteria emission between the studied seasons.

However, this finding contradicts a strong-quality study done by Gotkowska et al. (33) that was conducted in the same country but at different WWTP locations. This study found a significant difference in bacteria concentration presented in the air, depending on the sampling season. There was a negative correlation between the number of staphylococci and air humidity ($r = -0.286$, $p < 0.05$) as the air humidity was significantly varied between the seasons. It identified about 25 species of microorganisms in the WWTP air. Again, the mechanical sewage treatment produced substantial emissions of microorganisms into the air. However, it is shown that the number of microorganisms emitted was low if the process utilized fine bubble aeration.

Later, a moderate-quality study conducted by Oplinger et al. (34) used both personal and stationary air sampling and measured indoor and outdoor air at an STP. They found all bioaerosol concentrations to be above the recommended allowable limit of occupational exposure and varied with job tasks. Besides that, the enclosed areas' sewage processes had higher bacteria concentrations compared with the unenclosed areas. In contrast, Prazmo et al. (40) discovered that the airborne endotoxin concentration at the STPs located in eastern Poland was low and within the range of 0.1–5.2 ng/m^3. On top of that, fungi were identified in most of the samples along with bacteria. There was no significant correlation between microorganisms and endotoxin concentration. However, the findings were questionable as this study did not control any confounders, such as seasonal variation that might affect the concentration of airborne endotoxins at the STP.

Inhalable dust also was found to be present at an STP (36). For personal exposure, mechanics and sludge workers were exposed to a higher concentration of inhalable dust although, for stationary air assessment, the highest dust concentrations were found during the sludge dewatering process. The effect of climate variability over inhalable dust concentration was only explained in a small amount (1–7%). Overall, the inhalable dust and endotoxin exposure levels in Dutch STP were relatively low.

Others Hazardous Substance Concentrations in the Air

A strong-quality study conducted by Yang et al. (39) established hazardous substances other than endotoxins in the air at a WWTP in China. The authors were able to detect three major anions: nitric oxide, sulfur dioxide, and chloride. The anions can mostly be found in aerated grit chambers and anaerobic tanks. Regarding the endotoxin concentration, it was significantly higher at internal sites of the WWTP than external sites of WWTP, which heightens the inhalation risk magnitude among WWTP workers toward the microorganisms. Furthermore, the inhalation risk recorded was highest during summer compared with other seasons.

VOC Concentration in the Air

A study measured VOC concentrations in the atmosphere at the WWTP in China and surrounding areas (38). In this strong-quality study, it was found that, during summer, the VOC concentration recorded the highest reading. Interestingly, VOC not only can be found in the WWTP atmosphere, but can also travel to the surrounding area ∼4 km in radius. The primary clarifier site had the highest VOC concentration during summer. Among the VOC substances found in the study, benzene is the most readily released into the atmosphere.

DISCUSSION

This systematic review found that studies were done among human subjects in determining the association between potential respiratory hazard exposure and respiratory health among sewage workers, and they were diverse in design and sample sizes. It is difficult to draw solid conclusions due to the diversity in methodological and multiple exposures that could contribute to the respiratory health effects at the same time, such as endotoxins and chemicals. Also, some studies did not control the confounding factors and sometimes had incomplete control. Potential confounders found were age, smoking habit, obesity, gender, use of respirators, and previous history working in the dust industry. Also, self-selection bias by chance might produce

an association of respiratory hazards with poor respiratory health outcomes.

Most of the studies utilized a set of questionnaires to assess the presence or development of respiratory symptoms. Spirometry was used to measure pulmonary function. Next, for exposure assessment, the researchers preferred to use personal rather than stationary air sampling to measure the subjects' respiratory hazard exposure. Some studies were conducted extensively using invasive methods, such as taking blood samples, for instance, for polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), to determine the inflammation reaction in the subject's body.

From this review, it is reported that there are plenty of respiratory hazards that can be found at sewage plants. Thus, sewage workers are exposed to various hazards, including bioaerosols, chemicals, or both. The most studied risks were bioaerosols, which include endotoxins, bacteria, and fungi. Most of the studies report that endotoxin exposure could produce significant respiratory health effects among sewage workers in developing respiratory symptoms and a reduction in pulmonary function. The endotoxin concentration in the air differed between different worksites and processes. It is revealed that higher concentrations were identified at the sewage sludge treatment area.

However, a small number of studies found exposure to endotoxins or bioaerosols among sewage workers is not significantly associated with the development of adverse respiratory health effects (22, 27, 29). One of the reasons could be the lower geometric mean of endotoxin exposure among the sewage workers reported in those studies. Therefore, the sewage workers did not have a significant dose of exposure to produce negative respiratory health effects. On top of that, it is reported that the healthy worker effect phenomenon found during the study might be caused by selection bias among the subjects involved in the study (29).

H_2S was also found to be a potential respiratory hazard among sewage workers. Exposure to H_2S may cause deterioration in workers' pulmonary function and the development of respiratory symptoms. An invasive study approached using mean sulfhemoglobin% to determine the magnitude of H_2S exposure. It was found to be significantly higher among exposed workers compared to nonexposed workers. Even though several studies report that H_2S concentration in the air was moderate or lower than TLV of 1 ppm, the studies managed to find a significant association between H_2S exposure and the presence of respiratory symptoms among WWTP workers. Usually, sewage workers were more exposed to the incidental H_2S peak type of exposure than constant exposure. H_2S was also found to cause negative respiratory effects and cause central nervous system–related symptoms, such as tiredness and concentration difficulties among the sewage workers (47).

There are also studies other than at sewage plants that prove the significant association between H_2S exposure and adverse respiratory health effects. These studies were conducted at hog operations, housing nearby hog manure lagoons, and in the oil and refineries industries (48–50). However, a study done by Melbostad et al. (22), by contrast, did not find any significant relationship between H_2S exposure and the presence of respiratory symptoms. This finding parallels other studies done among pulp mill workers by Jappinen et al. (51). One of the reasons behind this was the duration of exposure among the workers. This means that workers exposed for a shorter duration of time to H_2S may not develop respiratory symptoms or reduce lung function. Another study conducted among 1,204 participants in a community based at the geothermal field in New Zealand had similar findings (52). The authors found that there was no significant association between long-term exposure of ambient H_2S concentration and lung function decrement or increased risk of chronic obstructive pulmonary disease (COPD) or asthma.

Furthermore, the H_2S exposure may benefit the lung function as it promotes airway smooth muscle relaxation. However, the authors realized that the findings could be affected by selection bias and exposure misclassification effects. Even though both of these studies were not conducted in the sewage plants, the evidence on H_2S exposure could not give rise to adverse respiratory effects should it be taken into consideration. The toxic effects of H_2S are characteristically dose-related, and its impact depends on the frequency and duration of exposure of the individual (53).

Inhalable dust is one of the potential hazards that can be inhaled easily by sewage workers if no proper personal protective equipment (PPE) is applied. Most of the researchers usually studied both inhalable dust and airborne bacteria concentration. It was found that higher inhalable dust concentrations produce a higher concentration of endotoxins (16, 19). The inhalable dust is mostly created during the aeration process in addition to the presence of inhalable dust, endotoxins, bioaerosols, and H_2S. Other chemical substances might contribute to developing respiratory symptoms among sewage workers, such as NH_3, NO, HCHO, and SO_2. Nonetheless, only one study managed to prove their presence and its effect on sewage workers' respiratory health (25).

Moving to the environmental assessment studies done at sewage plants, the researchers wanted to measure the concentration of the potential hazards at sewage plants and not determine the effect of the exposure on the subjects' respiratory health. Most of these studies used stationary air samplers rather than personal air samplers to quantify potential respiratory hazards. H_2S, bioaerosols (bacteria, fungi, and virus), PM 2.5, and VOC derivatives were mainly detected in the air at sewage plants. We found that more than half of environmental assessment studies in this systematic review need to consider the air samples in different seasonal variations. Seasonal factors play a significant role in humidity changes that could cause an alteration in potential hazard concentration, mostly airborne bacteria. Also, job locations or sites are among the main factors associated with respiratory hazard concentrations in the air. For example, an enclosed-type sewage plant may have a higher concentration of bacteria than an open-air type of sewage plant.

The possible reasons behind this finding may be that the higher airflow rate, leading to higher dilution effects of the bacteria, subsequently may lower the airborne bacteria concentration in the air. Next, sewage workers working within

the mechanical treatment process had a higher probability of being exposed to airborne bacteria. These findings show that the types of jobs are one of the principal associated factors that need to be considered to assess sewage workers' exposure levels.

To date, there has only been a small number of studies measuring H_2S, PM 2.5, and VOC concentration in sewage plant air. All these substances are proven in other studies to cause significant adverse respiratory health effects in industrial or occupational environments (15, 21–23, 25, 54–57). For example, in a study to estimate health risk from VOC removal from WWTP in China (38), the authors found a significant public health risk of VOC exposure to the people who live nearby the WWTP. The concentration of VOC emission is the highest at WWTP, which could harm the WWTP workers' respiratory health. In the future, there is a rising need to study VOC and particulate matter exposure among sewage workers and its effects on respiratory health. We can further discover new findings and valuable input to enhance knowledge gaps in this area.

LIMITATIONS

We encounter several limitations. First we were only able to identify a small number of studies related to the relationship between potential occupational hazard exposure at sewage plants and respiratory health effects among sewage workers. Thus, it is difficult to find a higher number of high-quality studies pertaining to this field. Next, we were unable to conduct a statistical meta-analysis study due to the variety of methodologies that have been applied in the studies, and this could have produced a more reliable conclusion. Although we have done a quite extensive literature search from five online databases and used inclusive search terms, we were unable to rule out the likelihood of failing to spot some relevant articles.

CONCLUSION

In conclusion, we found diverse literature in terms of study designs and results in this review. Overall, we need to accept an abundance of occupational respiratory hazard substances found at sewage plants, which could heighten the risk of developing adverse respiratory health effects among sewage workers. Several occupational respiratory hazards identified at sewage plants include H_2S, bioaerosols (endotoxins, fungi, bacteria, and virus), PM 2.5, and VOC. Nonetheless, a few studies produce insignificant or mixed results. Hence, there is still a great need to conduct additional studies in the future to identify potential new exposures of occupational respiratory hazards. These studies would also require more vigilant methodologies to clarify the short- and long-term respiratory health effects.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KM conceptualized this study, reviewed the literature, assessed the literature quality, and drafted the article. SMY, ZI, and ARI proposed an article outline, reviewed the literature, assessed the literature quality, and revised and polished the article. All authors contributed to the article and approved the submitted version.

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Comparative Evaluation of the Cytotoxicity of Glyphosate-Based Herbicides and Glycine in L929 and Caco2 Cells

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Introduction: Glyphosate, an amino acid analog of glycine, is the most widely applied organophosphate pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including the formulation "Roundup. " While glycine is an essential amino acid generally recognized safe, both epidemiological and toxicological *in vivo* and *in vitro* studies available in literature report conflicting findings on the toxicity of GBHs. In our earlier *in vivo* studies in Sprague–Dawley rats we observed that exposure to GBHs at doses of glyphosate of 1.75 mg/kg bw/day, induced different toxic effects relating to sexual development, endocrine system, and the alteration of the intestinal microbiome. In the present work, we aimed to comparatively test in *in vitro* models the cytotoxicity of glycine and GBHs.

Methods: We tested the cytotoxic effects of glycine, glyphosate, and its formulation Roundup Bioflow at different doses using MTT and Trypan Blue assays in human Caco2 and murine L929 cell lines.

Results: Statistically significant dose-related cytotoxic effects were observed in MTT and Trypan Blue assays in murine (L929) and human (Caco2) cells treated with glyphosate or Roundup Bioflow. No cytotoxic effects were observed for glycine. In L929, Roundup Bioflow treatment showed a mean IC50 value that was significantly lower than glyphosate in both MTT and Trypan Blue assays. In Caco2, Roundup Bioflow treatment showed a mean IC50 value that was significantly lower than glyphosate in the MTT assays, while a comparable IC50 was observed for glyphosate and Roundup Bioflow in Trypan Blue assays. IC50 for glycine could not be estimated because of the lack of cytotoxic effects of the substance.

Conclusion: Glyphosate and its formulation Roundup Bioflow, but not glycine, caused dose-related cytotoxic effects in *in vitro* human and murine models (Caco2 and L929). Our results showed that glycine and its analog glyphosate presented different cytotoxicity profiles. Glyphosate and Roundup Bioflow demonstrate cytotoxicity similar to other organophosphate pesticides (malathion, diazinon, and chlorpyriphos).

Keywords: mechanism, glyphosate, cancer, toxicity, biomarker

INTRODUCTION

Glyphosate [IUPAC chemical name N-(phosphonomethyl)-glycine], an amino acid analog of glycine, is the most widely applied organophosphate pesticide worldwide and it is an active ingredient of all glyphosate-based herbicides (GBHs), including in the formulation "Roundup" (1, 2). It is mainly marketed as a broad-spectrum systemic herbicide and crop desiccant (3). Glyphosate was in fact synthesized in 1950 by a Swiss chemist, Henri Martin, as an analog of the non-essential amino acid glycine, but its herbicidal properties were not discovered for another 20 years (4). The massive and increasing use of GBHs leads to a global burden of occupational exposures in manufacturing workers and GBH applicators (farmers), as well as increasing exposures in the general population, as demonstrated by environmental contamination from glyphosate residues found in air (5), groundwater (6, 7), drinking-water (8), crops (9, 10), food (11, 12), and animal feed (13). In humans, the main exposure routes to glyphosate are inhalation and dermal exposure in the occupational setting and for the general population consumption of contaminated drinking water and residues in food items (14).

The results of oral studies with [^{14}C] glyphosate in rats, rabbits and goats indicate that absorption from the gastrointestinal tract is incomplete and amounts to up to 30% of the dose (15–17). The most relevant routes of excretion following oral administration of glyphosate [^{14}C] are feces (70–80%) and urine (20–30%) (18). Therefore, most of the glyphosate assumed orally is not absorbed in the gastro-intestinal tract and is then excreted with the feces. On the other hand, glycine is very rapidly absorbed along the gastrointestinal tract via special carrier systems and then transported via the portal vein into the liver but also distributed within the whole body since it is involved in the body's production of haem, DNA, phospholipids, and collagen (19).

In March 2015, the World Health Organization's International Agency for Research on Cancer (IARC) classified three organophosphates (glyphosate, malathion, and diazinon) as "probably carcinogenic for humans" (Category 2A) (20). In contrast, in November 2015 the European Food Safety Agency determined glyphosate was "unlikely to pose a cancer risk for man" (EFSA 2015). In 2018 the European Chemicals Agency (21) Risk Assessment Committee concluded that "the scientific evidence so far available does not satisfy the criteria for classifying glyphosate as carcinogenic, mutagenic or toxic for reproduction" (21). In 2019 a US federal health agency, the Agency for Toxic Substances and Disease Registry (ATSDR) (22), part of the Centers for Disease Control and Prevention (23), determined that both cancer and non-cancer hazards derive from exposure to glyphosate and GBHs. The ATSDR 2019 report clearly lays out the vast array of scientific evidence linking both pure glyphosate (rodent studies) as well as formulations (in human epidemiologic studies) to cancer. In fact glyphosate, as the pure active substance, and its formulations may not have the same toxicity. Glyphosate formulations contain a number of so-called "inert" ingredients or adjuvants to facilitate the uptake by plants, most of which are patented and not publicly known (in many countries the law does not require a full disclosure of pesticide ingredients). GBHs that contain surfactants and adjuvants might act differently than glyphosate alone (24–26). In fact, adjuvants might be toxic in their own right and potentiate the toxic effects of glyphosate, as in the case of polyethoxylated tallow amine (POEA), that have been banned in the EU since 2016 (27–31).

While glycine is an essential amino acid generally recognized safe, both epidemiological and toxicological *in vivo* and *in vitro* studies available in literature report conflicting findings on the toxicity of GBHs. In our previous *in vivo* studies on Sprague–Dawley rats we observed that exposure to GBHs (pure glyphosate and Roundup Bioflow) at doses of glyphosate considered to be "safe," the US ADI of 1.75 mg/kg bw/day, defined as the chronic Reference Dose (cRfD) determined by the US EPA, induced different toxic effects relating to sexual development, endocrine system, and the alteration of the intestinal microbiome (32–34). Furthermore, mechanistic data are increasingly important for hazard characterization, as exemplified by the 10 key characteristics of carcinogens considered by IARC in their evaluations, which include "Alters cell proliferation, cell death or nutrient supply" (35, 36). In particular, the MTT and Trypan Blue assays are routine and convenient methods for determination of cytotoxicity (37–39). MTT assay is a colorimetric assay of viable cells, while Trypan Blue assay is a dye exclusion staining assay. The combined and comparative use of MTT and Trypan Blue assays allows to overcome the limitations the single assays and increases the precision of the IC50 estimates derived from these studies (40–42). In the present work, we aimed to test the cytotoxic effects of glycine, glyphosate and its formulation Roundup Bioflow at different doses using MTT and Trypan Blue assays in human Caco2 and murine L929 cell lines.

MATERIALS AND METHODS

Cell Cultures

L929 mouse fibroblasts (ATCC-CCL1) were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco) added with 10%, fetal bovine serum (FBS, Gibco), 1 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Caco2 human epithelial cell line (ATCC HTB-37) obtained from colorectal adenocarcinoma were cultured with DMEM with 10%, of FBS and 1% penicillin-streptomycin. Cells were grown in a monolayer condition at 37°C in an atmosphere of 5% CO_2 and for the cell treatments, cells were cultured with DMEM alone.

Test Substance

Glyphosate (Pestanal^{TM} analytical standard, CAS number 1071-83-6, purity > 99.5%) was obtained from Sigma-Aldrich (Milan, Italy). The commercial formulation Roundup Bioflow (containing 360 g/L of glyphosate acid in the form of 480 g/l isopropylamine salts of glyphosate (41.5%), water (42.5%), and surfactant (16%, ingredient not disclosed by the producer) was supplied from a local agricultural consortium (Consorzio Agrario dell'Emilia, Bologna, Italy). Glycine was supplied from Biosolve (Valkenswaard, Netherlands).

Cell Treatments

Glycine (Biosolve), Glyphosate (Sigma-Aldrich), and Roundup®Bioflow were added to cells for 24 h. Glycine and Glyphosate were diluted in water and the treatment doses were prepared at different concentrations according the following formula:

$$175\ mg/kg\ bw\ (US\ NOAEL) \times 70\ kg\ (average\ body\ weight\ of\ an\ adult)/2\ Lt\ (daily\ water\ intake)$$

The final concentration of the treatments was of 6.125 g/L [36.676 mmol/L]; 0.6125 g/L [3.667 mmol/L]; 0.06125 g/L [0.3667 mmol/L], and 0.006125 g/L [0.03667 mmol/L] (equivalent respectively to 175, 17.5, 1.75, and 0.175 mg/kg bw). Each dilution was prepared in DMEM and the pH of each final solution was corrected to 7.0 by adding $NaHCO_3$ (Gibco).

Measurement of Cell Proliferation for Adherent Cells (MTT)

Cell cytotoxicity was measured using the3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) assay. L929 cells (5×10^4 cells/well) and Caco2 cells (10^5 cells/well) were plated in 96-well tissue culture plate in complete medium (100 µL/well). The multiwell plates were incubated at 37°C, 5% CO_2 for 24 h. After 24 h, the culture medium was removed and equal volumes (100 µL) of the treatments were added to each well. In control wells, 100 µL DMEM were added. Control wells consisted of untreated cell cultures. Twenty-four hours later, proliferative cells were detected by MTT assay, according to the ISO 10993-5 International Standard procedure (43). The main purpose of the ISO 10993-5 procedure is to define a scheme for testing *in vitro* cytotoxicity of different extracts according to a multi-step approach. Briefly, cells were incubated with MTT solution (1 mg/mL, Life Technologies) at 37°C for 2 h. Then, MTT solution was removed and cells were solubilized with 100 µl of isopropanol. The formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm. The results were expressed as percentage of viable cells compared to controls.

Measurement of Cell Viability (Trypan Blue) and Average Cell Size

Cell viability was measured using the Trypan Blue assay. L929 and Caco2 cells were plated in 24-well tissue culture plate (50×10^5 cells/well) in complete medium. The day after, treatments diluted in DMEM were added to cells for 24 h. To detect viability and cells size, cells were trypsinized with a solution of trypsin 0.05% and EDTA 0.02%. Cell were then carefully diluted in a 0.4% Trypan Blue (Gibco) solution by preparing a 1:1 dilution with the cell suspension. Viable cells were counted and average cell size analyzed by using Countess®II FL (Thermo Fisher Scientific). The results were expressed as percentage of viable cells compared to controls.

Statistical Analysis

The MTT cell tests were carried with six replicates for each treatment and data were expressed as mean values of three different experiments. Statistical analysis was performed with R software (44). Normal and homoscedastic data were analyzed with ANOVA followed by pairwise comparison (Dunnett test) and Tukey *post-hoc* tests with Bonferroni correction. Non-normal homoscedastic data were analyzed with the non-parametric Kruskall–Wallis test and Dunn's *post-hoc* test with Bonferroni correction. Differences were considered to be significant at a $p < 0.05$. IC50 values were calculated by Probit regression.

RESULTS

MTT: Effects of Glycine, Glyphosate, and Roundup Bioflow on L929 and Caco2 Cells Proliferation

Glycine did not modulate either L929 or Caco2 cell proliferation at any of the concentrations used in the MTT assays (**Figure 1**). Statistically significant decreases of proliferating cells were observed at all doses of glyphosate compared to controls in both L929 models and Caco2 models, except at the lowest dose in Caco2 (**Figure 1**). Glyphosate showed also positive correlation between the percentage of proliferating cells as a function of the concentration for both L929 cells ($R = 0.957$, **Figure 2A**) and Caco2 cells ($R = 0.986$, **Figure 2B**). Statistically significant decreases of proliferating cells were observed at all doses of Roundup Bioflow compared to controls in both L929 models and Caco2 models, except at the lowest dose in L929 (**Figure 1**). Roundup Bioflow showed also positive correlation between the percentage of proliferating cells as a function of the pesticide concentration for both L929 and Caco2 cells, with $R = 0.956$ and 0.978, respectively (**Figures 2A,B**).

Trypan Blue: Effects of Glycine, Glyphosate, and Roundup Bioflow on L929 and Caco2 Cells Viability

Glycine did not modulate either L929 or Caco2 cell viability at any of the concentrations used for cell treatments (**Figure 1**). Statistically significant decreases of vital cells were observed at all doses of glyphosate compared to controls in both L929 models and Caco2 models (**Figure 1**). Glyphosate showed also a positive correlation between the percentage of vital cells compared to control and the dose treatments both for L929 fibroblasts ($R = 0.978$, **Figure 3A**) and intestinal cell Caco2 ($R = 0.972$, **Figure 3B**). Statistically significant decreases of vital cells were observed at all doses of Roundup Bioflow compared to controls in both L929 models and Caco2 models (**Figure 1**). Roundup Bioflow showed also a positive correlation as a function of the pesticide concentration for both L929 and Caco2 cells with $R = 0.975$ (**Figure 3A**) and $R = 0.996$ (**Figure 3B**), respectively.

Effects of Glyphosate and Roundup Bioflow on L929 and Caco2 Average Cell Size

Statistically significant decreases of average cell size were observed at all doses of glyphosate compared to controls in both L929 models and Caco2 models (**Figure 4**). Statistically significant decreases of average cell size were observed at all doses

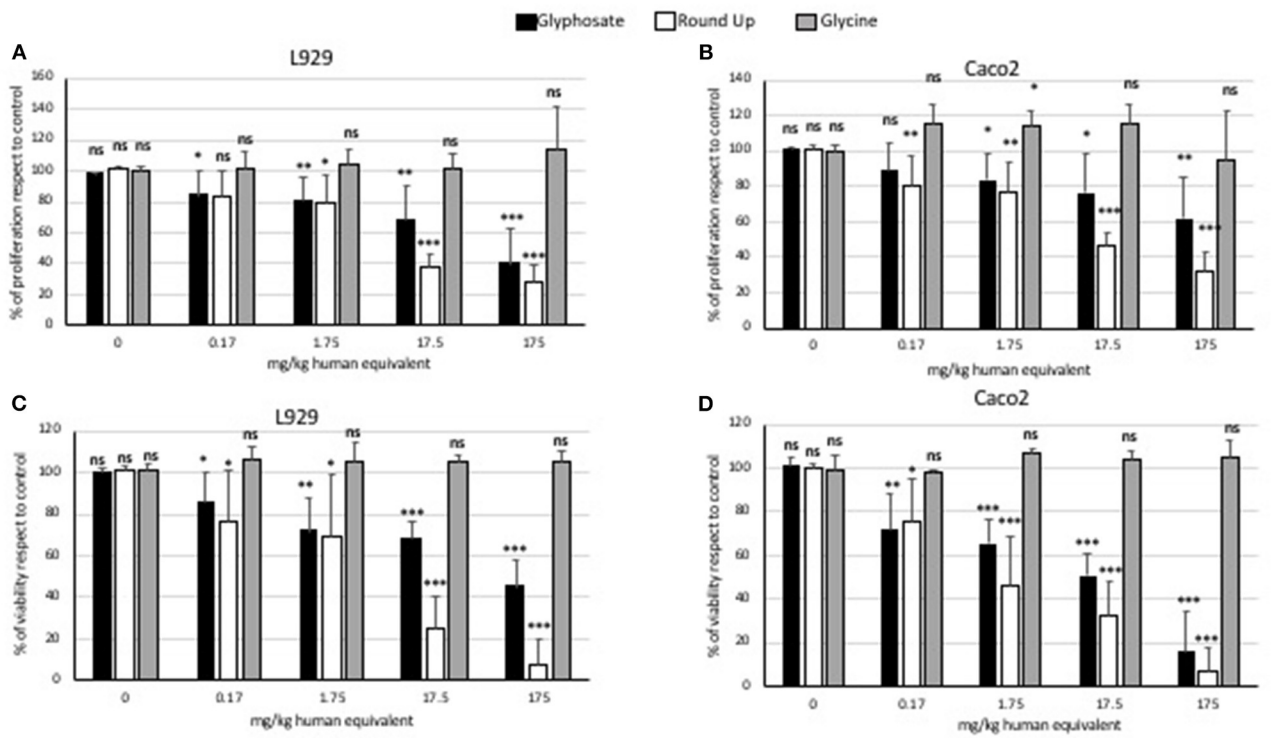

FIGURE 1 | MTT (proliferation) assay and Trypan Blue (viability) assay: effects of pure glyphosate, Roundup Bioflow, and glycine at the dose range 0–175 mg kg^{-1} (human equivalent) on cell proliferation of L929 fibroblasts **(A)**, Caco2 cells **(B)**, and on cell viability of L929 fibroblasts **(C)** and Caco2 cells **(D)**. Data are expressed as mean value (± st. dev.) (% compared to control). Pairwise comparison based on Anova (Dunnett test). ns, not significant: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

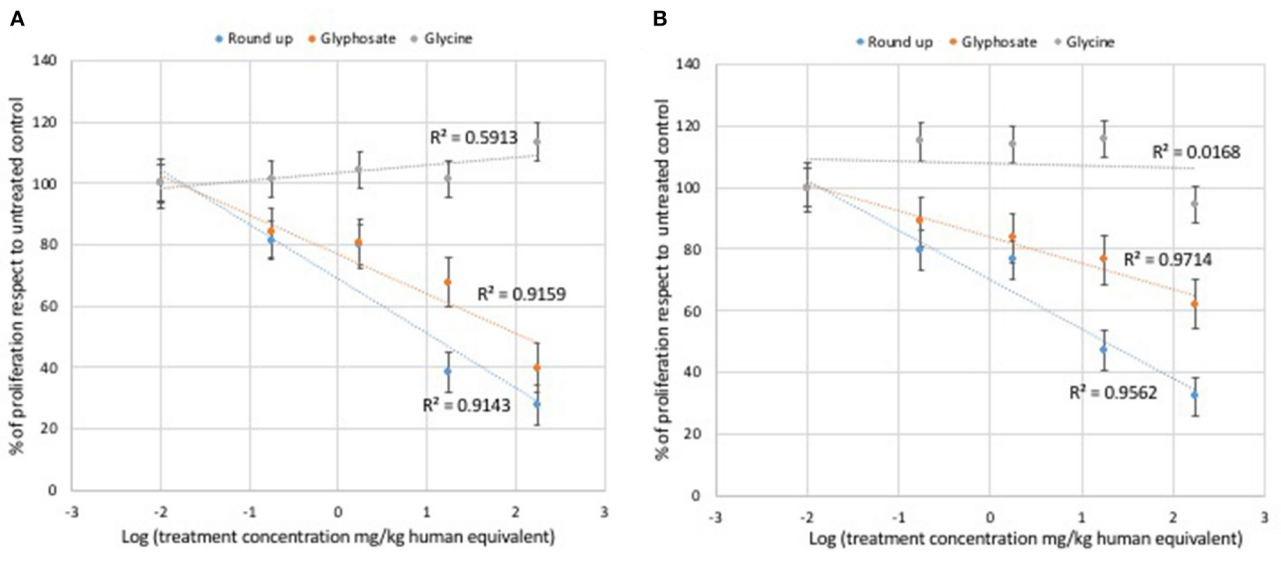

FIGURE 2 | Relationship between pure glyphosate, Roundup Bioflow, and glycine at the dose range 0–175 mg kg^{-1} (human equivalent) (Log scale) and cell proliferation of L929 fibroblasts **(A)** and Caco2 cells **(B)**. Data are expressed as mean value (± st. dev.) (% compared to control). R^2, coefficient of determination.

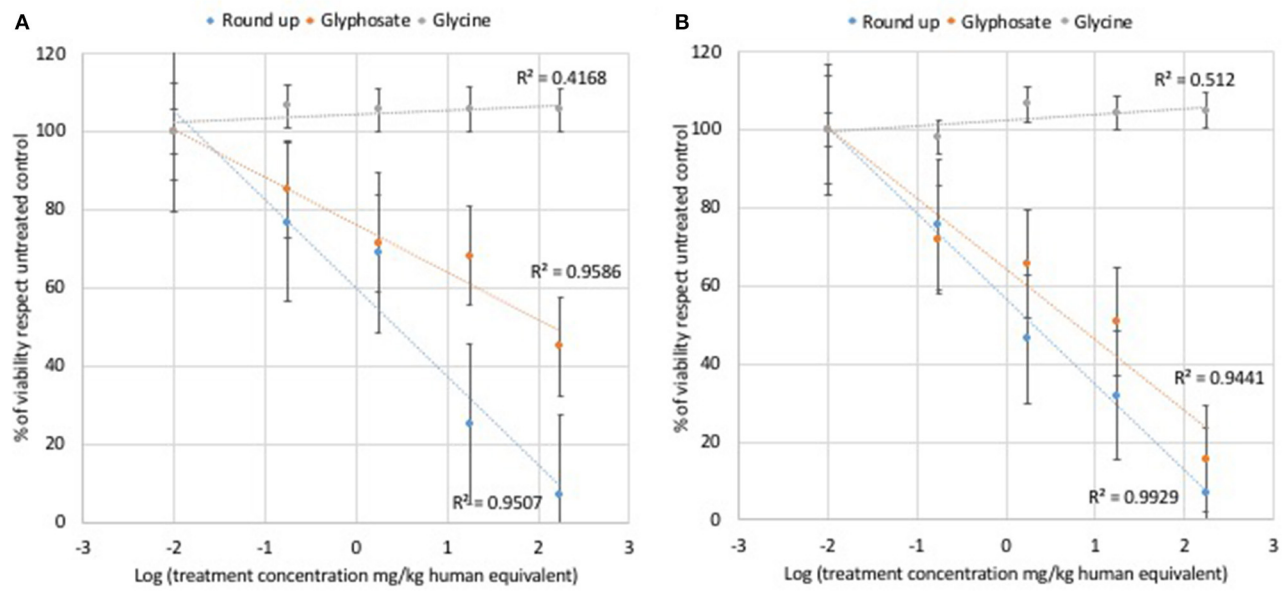

FIGURE 3 | Relationship between pure glyphosate, Roundup Bioflow, and glycine at the dose range 0–175 mg kg^{-1} (human equivalent) (Log scale) and cell viability of L929 fibroblasts **(A)** and Caco2 cells **(B)**. Data are expressed as mean value (± st. dev.) (% compared to control). R^2, coefficient of determination.

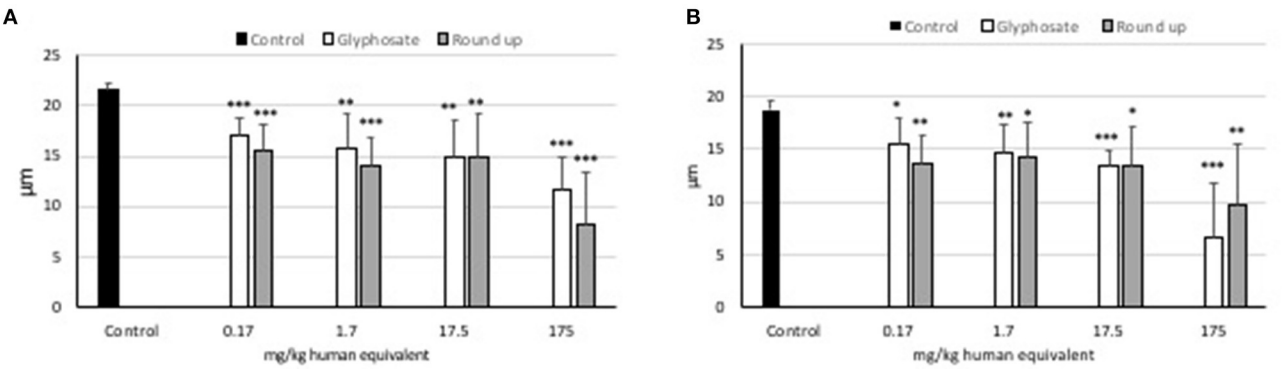

FIGURE 4 | Effects of pure glyphosate and Roundup Bioflow at the dose range 0–175 mg kg^{-1} (human equivalent) on cell size of L929 fibroblasts **(A)** and Caco2 cells **(B)**. Data are expressed as mean value (± st. dev.). Pairwise comparison based on Anova (Dunnett test). ns, not significant: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of Roundup Bioflow compared to controls in both L929 models and Caco2 models (**Figure 4**).

Correlation Between MTT and Trypan Blue Assays for L929 and Caco2 Cells Treated With Glyphosate and Roundup Bioflow

A correlation was performed between results obtained from the MTT assay and the viability analysis obtained by the treatment of L929 (**Figure 5**) and Caco2 cells (**Figure 6**) with Glyphosate and Roundup Bioflow. Regarding L929 fibroblasts, both Glyphosate (**Figure 5A**) and Roundup Bioflow (**Figure 5B**) treatments showed a positive correlation as a function of the concentrations, even if it resulted more evident when L929 fibroblasts were treated with Roundup Bioflow. Regarding Caco2 cell treatments, both Glyphosate (**Figure 6A**) and Roundup Bioflow (**Figure 6B**) treatments showed a positive correlation as a function of the concentrations even if it resulted more evident when L929 fibroblasts were treated with Roundup Bioflow. Analysis revealed that both of the correlations were statistically significant at $P < 0.05$.

IC50 (ug/L) Mean Values Calculated for MTT and Trypan Blue on L929 and Caco2 Cell Lines

IC50 mean values for MTT and viability and size regarding the treatments of Glycine, Glyphosate, and Roundup Bioflow was calculated related L929 fibroblasts and Caco2 cells. For Glycine, it was not possible to calculate IC50 for either L929

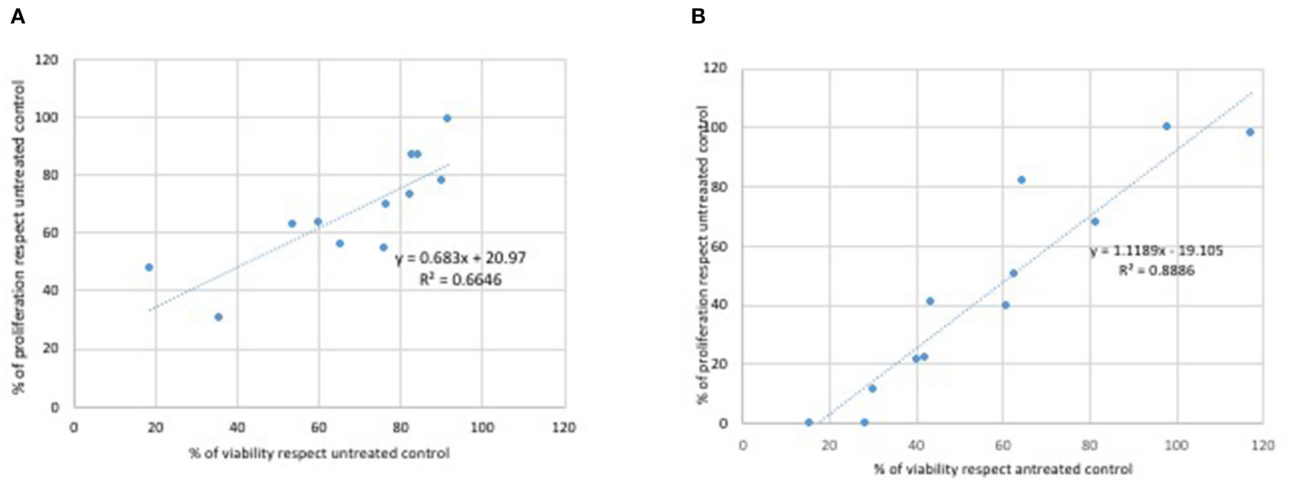

FIGURE 5 | Correlation between MTT and Trypan Blue test for L929 cells treated with different doses of Glyphosate **(A)** and Roundup **(B)**. Both correlations were statistically significant at $P < 0.05$.

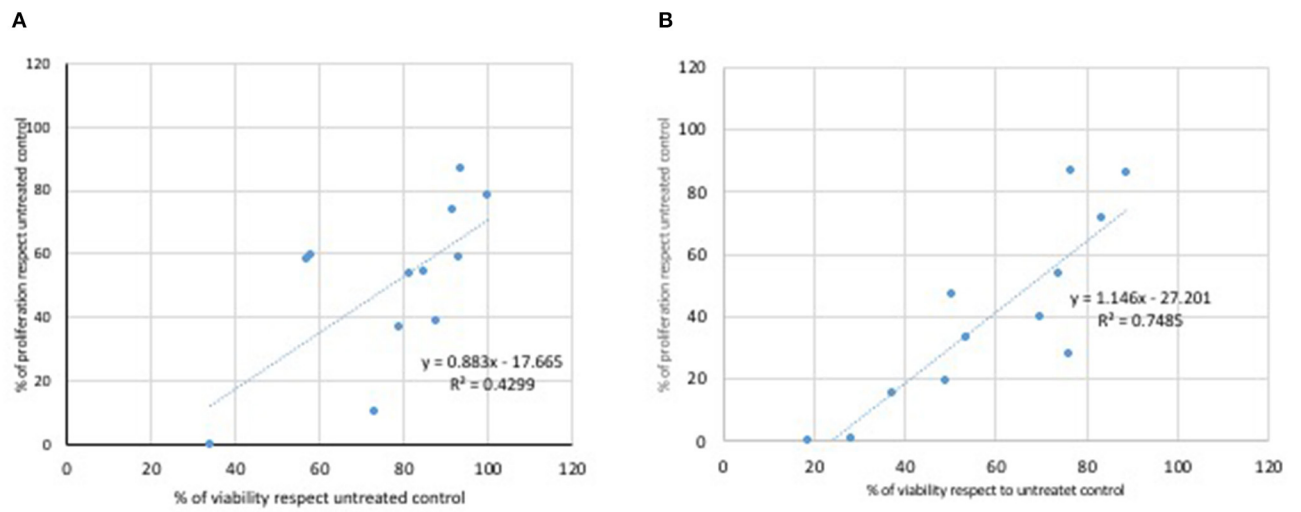

FIGURE 6 | Correlation between MTT and Trypan Blue test for Caco2 cells treated with different doses of Glyphosate **(A)** and Roundup **(B)**. Both correlations were statistically significant at $P < 0.05$.

fibroblasts or Caco2 cells. In L929 treatments (**Table 1**) Roundup Bioflow showed IC50 mean value significantly lower compared to the IC50 mean value induced by Glyphosate treatments for both the MTT and Trypan Blue assays. In Caco2 treatments (**Table 2**), Roundup Bioflow showed IC50 which was significantly lower than the one induced by Glyphosate for MTT, while statistical significance was not reached for differences relating viability assay.

TABLE 1 | IC50 (μg/L) mean values calculated for MTT, viability on L929 cells.

	MTT	Viability
Glycine	ND	ND
Glyphosate	119.9 ± 38.5 (a)	147.9 ± 22.4 (a)
Roundup Bioflow	11.7 ± 5.3 (b)	2.8 ± 2.3 (b)

One way Anova (different letters indicates statistically different mean values at P < 0.05).

DISCUSSION

The MTT and viability results confirm a different mechanism of action of glycine and its analog glyphosate (and its formulation Roundup Bioflow). In fact glycine did not show any sign of cytotoxicity, confirming the extremely safe profile of this substance, in line with the reported EU NOAEL (2,000 mg/Kg bw). On the other hand, glyphosate and its formulation Roundup showed a clear dose-related cytotoxic effects in MTT and viability assays in both Caco2 human intestinal cell line and L929 murine

TABLE 2 | IC50 (μg/L) mean values calculated for MTT, viability on Caco2 cells.

	MTT	Viability
Glycine	ND	ND
Glyphosate	ND	6.2 ± 3.5 (a)
Roundup Bioflow	18.1 ± 6.3 (b)	1.9 ± 1.1 (a)

One way Anova (different letters indicates statistically different mean values at $P < 0.05$).

fibroblast cell line. The cytotoxic effects were also observed at doses that are lower than the current EU NOAEL (50 mg/Kg bw). Therefore, our results confirm previous evidence of cytotoxicity of glyphosate in *in vitro* models on Raji human hematological cell lines (45). In addition, our findings support the hypothesis of a higher toxic potency of the formulation, compared to pure glyphosate, in line with the results observed in other *in vitro* cytotoxicity studies by other authors (28, 46) and the results of our recent *in vivo* studies on Sprague–Dawley rats exposed to the same formulation (Roundup Bioflow) (34). Furthermore, different organophosphates other than glyphosate proved had similar effects in different *in vitro* models: in particular malathion exposure induced dose-dependent cytotoxic effects in MTT assays performed on HepG2 human liver cell line (47), diazinon exposure induced dose-dependent cytotoxic effects in MTT assays performed on HCT116 human intestinal cell line (48), chlorpyrifos induced dose-and time-dependent cytotoxic effects in MTT assays performed on SH-SY5Y human neural cell line (49). However, our experiment presents certain limitations: (1) direct quantitative extrapolations of IC50 values from cell lines to the human *in vivo* situation might under or overestimate the effects, therefore further studies are recommended to establish a dose-response relationship *in vivo* (50) (2) the equivalent dose was calculated based on the assumption that all of the substance administered would lead to exposure of the intestinal system (in the case of the intestinal model Caco2) or in a distant organ (the case of the fibroblast model L929), however the first is a probably more realistic exposure scenario for glyphosate in light of the relatively limited systemic absorption of glyphosate (and the high level of gastrointestinal exposure) (17), while the second might be a more realistic exposure for glycine that presents higher systemic absorption rates; (3) routes of exposures other than oral that are particularly relevant in the occupational setting, thus inhalation and/or skin absorption, were not taken into account in this model, although glyphosate can reach through this routes other organs and systems before being metabolized to AMPA; (4) we could not test separately the cytotoxicity of the adjuvants present in the formulation Roundup Bioflow as these are trade secrets.

CONCLUSION

Glyphosate and its formulation Roundup Bioflow, but not glycine, caused dose-related cytotoxic effects in *in vitro* MTT and viability assays in human intestinal and murine fibroblast models (Caco2 and L929). Our results showed that glycine and its analog glyphosate presented different cytotoxicity profiles. Glyphosate and Roundup Bioflow demonstrate cytotoxicity similar to other organophosphates, in particular malathion, diazinon, and chlorpyriphos. Notably, glyphosate, diazinon, and malathion have been recently classified as probable carcinogen by IARC (Group 2A) (51). The formulation Roundup Bioflow seem to be more cytotoxic than pure glyphosate. Cytotoxic effects of GBHs were observed at doses that are lower than the current EU NOAEL (50 mg/Kg bw).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DM and FT prepared the first draft of manuscript. DM, FT, and GD designed the experiments. FT conducted the experiments. GD and FB supervised the work. GD performed statistical analyses and reviewed the manuscript. PS, ES, and FG critically revised the manuscript and originally contributed to the methods and the elaboration of the results. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Public Health

BRIEF RESEARCH REPORT
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Short Report: Using Targeted Urine Metabolomics to Distinguish Between Manganese Exposed and Unexposed Workers in a Small Occupational Cohort

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Objectives: Despite the widespread use of manganese (Mn) in industrial settings and its association with adverse neurological outcomes, a validated and reliable biomarker for Mn exposure is still elusive. Here, we utilize targeted metabolomics to investigate metabolic differences between Mn-exposed and -unexposed workers, which could inform a putative biomarker for Mn and lead to increased understanding of Mn toxicity.

Methods: End of shift spot urine samples collected from Mn exposed ($n = 17$) and unexposed ($n = 15$) workers underwent a targeted assay of 362 metabolites using LC-MS/MS; 224 were quantified and retained for analysis. Differences in metabolite abundances between exposed and unexposed workers were tested with a Benjamini-Hochberg adjusted Wilcoxon Rank-Sum test. We explored perturbed pathways related to exposure using a pathway analysis.

Results: Seven metabolites were significantly differentially abundant between exposed and unexposed workers (FDR ≤ 0.1), including n-isobutyrylglycine, cholic acid, anserine, beta-alanine, methionine, n-isovalerylglycine, and threonine. Three pathways were significantly perturbed in exposed workers and had an impact score >0.5: beta-alanine metabolism, histidine metabolism, and glycine, serine, and threonine metabolism.

Conclusion: This is one of few studies utilizing targeted metabolomics to explore differences between Mn-exposed and -unexposed workers. Metabolite and pathway analysis showed amino acid metabolism was perturbed in these Mn-exposed workers. Amino acids have also been shown to be perturbed in other occupational cohorts exposed to Mn. Additional research is needed to characterize the biological importance of amino acids in the Mn exposure-disease continuum, and to determine how to appropriately utilize and interpret metabolomics data collected from occupational cohorts.

Keywords: occupational health, exposure assessment, targeted metabolomics, manganese exposure, biomonitoring

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INTRODUCTION

Manganese (Mn) is a known neurotoxicant associated with a range of motor (1–3) and cognitive (4–6) health outcomes. Elevated exposure to Mn occurs most frequently in metal-working occupational settings, such as among solderers, welders, brazers, and foundry workers. While environmental Mn exposures are typically lower than in occupational settings, elevated environmental exposures can occur in proximity to Mn-utilizing industrial facilities or busy roadways, putting more people at risk for health outcomes related to Mn (7–9).

The gold-standard for measuring airborne Mn exposure remains filter-based personal air sampling, though a variety of exposure biomarkers have been explored in both occupational and environmental cohorts (10–14). However, notable limitations to using common biologic matrices, such as urine, blood, and plasma, to assess Mn exposure have been discussed in the literature (15). Magnetic resonance imaging (MRI) and positron emission tomography (PET) have been found to be promising assessment methods across a range of exposures; however, contraindications to these procedures and the cost and specialized equipment required can reduce their utility for routine use (16–18).

Given the limitations of common Mn exposure assessment methods, there is a public health interest in investigating readily accessible biomarkers related to Mn exposure, such as urinary metabolites that distinguish between exposed and unexposed persons. These small molecule metabolites could serve as putative biomarkers of Mn exposure and help elucidate the biological processes that Mn may perturb either simultaneously with or prior to exerting neurotoxicity. Metabolomics is the study of these small molecules (<1,500 Daltons) that are important in metabolic processes. Previously, we have utilized global metabolomics profiling to investigate metabolites that differ between exposed and unexposed workers in the Puget Sound region, whose data are also used in this manuscript (19). In our previous work, we found nine metabolites to be significantly differentially abundant between exposed and unexposed workers [false discovery rate (FDR) <0.1], and most of these metabolites also exhibited an exposure-response relationship when stratifying workers by no exposure, low exposure, and high exposure. However, when investigating these nine metabolites in a different occupationally exposed cohort of welders, these nine metabolites were no more predictive of exposure status than by chance alone (20). As this previous work utilized global metabolomics methods, the identity of the nine metabolites were not known, making it challenging to infer biologic relevance as to why they may not have replicated in another occupational cohort. To improve on this limitation, targeted metabolomics, where the identity of the metabolite is known, was utilized in the Puget Sound cohort to investigate not only metabolite differences between groups defined by exposure, but also potential pathway perturbations related to Mn exposure in these workers, which could inform how Mn exerts toxicity in exposed individuals.

METHODS

Study Population and Samples Collected

Foundry workers at a Mn-steel foundry and crane operators or truck drivers at a scrap metal recycling yard were organized into a meeting by their site health and safety officer. Here, workers were given an overview of the study by the study team, given a chance to ask questions, and interested participants were enrolled in our study after giving written, informed consent. All study protocols were approved by the University of Washington Institutional Review Board (IRB number 47550). A total of 20 Mn-exposed foundry workers and 17 Mn-unexposed crane operators and truck drivers were recruited into our study. Both workplaces are located in the Puget Sound region of Washington state. The characteristics of the cohort and details on the study design have been previously described by Baker et al. (19). In October 2014, a full-shift personal air sample was collected from these workers to ensure there was not exposure misclassification between the exposed and unexposed groups. Airborne Mn exposure was assessed using Institute of Medicine (IOM) inhalable dust samplers, which were analyzed for the inhalable Mn fraction according to the UK Health and Safety Executive's Methods for the Determination of Hazardous Substances 14/4 (21). The mean 8-h time weighted average (TWA) Mn exposure in the foundry workers was 365 $\mu g/m^3$ [standard deviation (SD): 300, range: 98.5, 1,243] whereas the mean Mn exposure for the crane operators/truck drivers was 9.2 $\mu g/m^3$ (SD: 36.5, range: 0.02, 150.8), confirming the expected Mn exposure difference between the exposed and unexposed workers. The mean 8-h TWA Mn exposure in the foundry workers exceeded the American Conference of Governmental Industrial Hygienist's (ACGIH) threshold limit value (TLV) of 100 $\mu g/ m^3$, while the mean exposure for the crane operators/truck drivers was well below this limit.

An end-of shift spot urine sample was collected from 17 Mn-exposed foundry workers and 15 unexposed crane operators or truck drivers in January 2015. Clean catch urine samples were collected in 125 mL wide mouth low-density polypropylene bottles (Nalgene). Urine samples were aliquoted on site into 2 mL Safe-Lock Eppendorf tubes, and immediately stored on dry ice for transport to University of Washington (UW) in Seattle, where they were stored at $-80^{\circ}C$ awaiting metabolomics analysis. Participants completed a short exposure questionnaire when their urine was collected in January 2015. The goal of this questionnaire was to confirm that current job duties were similar to job duties in October 2014 when personal airborne exposure had been assessed.

Targeted Metabolomics Analysis

A targeted assay of 362 aqueous metabolites was undertaken via liquid chromatography-tandem mass spectrometry (LC-MS/MS) on the urine samples. Sample preparation and analysis was completed by the Northwest Metabolomics Research Center at UW. Frozen urine samples were thawed on wet ice and vortexed. Next, 100 μL of urine were mixed with methanol in a 2:1 ratio to precipitate any residual protein, and a solution

containing 32 isotope-labeled internal standards was added to the urine-methanol mixture to monitor sample preparation and quantitate metabolites. After drying the samples using a Vacufuge (Eppendorf), samples were reconstituted in mobile phase B solvent (see below) and diluted by 5:1 prior to LC-MS/MS analysis.

These Puget Sound samples were randomized with urine samples from a separate study undergoing the same metabolomics analysis in a three batch run. A pooled quality control (QC) sample was constituted using small aliquots from the study samples, and it was run once for every ten study samples for a total of 12 pooled QC samples. In addition, a pooled instrument control sample (serum) was also run once every 10 study samples, along with blank samples. Samples were injected into the chromatography system (CDC autosampler and Shimadzu Nexera LC-20 pumps) consisting of a dual injection valve setup allowing injections onto two different LC columns with each column dedicated to an ESI polarity. For positive mode ionization, 5 μL were injected on to the column, and 10 μL on to the column for negative mode. Both columns were Waters XBridge BEH amide columns (2.1 × 150 mm) from the same production lot. The autosampler was maintained at 4°C and the column oven was set to 40°C. Mobile phase A was 10 mM ammonium acetate in 95% water, 3% acetonitrile, 2% methanol, and 0.2% acetic acid, and Mobile phase B was 10 mM ammonium acetate in 93% acetonitrile, 5% water, 2% methanol, and 0.2% acetic acid. The 0.3 mL/min solvent gradient was as follows: 0–1.5 min 95% mobile phase B, 1.5–6 min 95% to >70% B, 6–10 min 70% B, 10–12 min 70–45% B, 12–14 min 45% B, 14–15 min 45% to >95% B, 15–18 min 95% B. After completion of the 18 min gradient, injection on the other column was initiated and the inactive column was allowed to equilibrate at the starting gradient conditions. A set of QC injections for both instrument and sample QC were run at the beginning and end of the sample batch, as well as every 10 study samples. Blank samples were run periodically as well to monitor carryover.

The MS data were integrated using SCIEX MultiQuant 3.0.2 or Sciex-OS v1.5 software. Peaks were selected based on peak shape, a signal-to-noise ratio of >10, and retention times consistent with previously run standards and sample sets. The median metabolite coefficient of variation (CV) in the pooled QC samples over the course of the run was 7.9%; the median metabolite CV for the pooled laboratory standard QC samples over the course of the run was 7.8%.

Statistical Analysis

Metabolites which were not present above the limit of detection in at least 50% of the samples were removed, resulting in a total of 224 metabolites (62%) included in our sample set. Missing values were replaced with 1×10^{-6} prior to normalization and $\log_{10}$-transformation. In order to account for systematic errors resulting from instrument drift and differences in urine dilution and hydration between the participants, samples were normalized in Metaboanalyst 4.0 by the set of pooled QC samples (22), a step undertaken for each individual metabolite measured. This normalization step is important for reducing systematic variation and allowing true biological variation between the samples to be revealed (23). For this type of normalization, pooled probabilistic quotient normalization (PQN) is used, which looks at the distribution of metabolites across the pooled QC samples and adjusts the participant samples based on the QC samples, therefore relying on reference samples instead of the study samples themselves (23). This method also adjusts for differences in dilution by determining a probabilistic dilution factor for each sample, based on the differences between each sample and the pooled QC samples. After applying this normalization to the pooled QC samples, the median metabolite CV over the course of the run decreased to 4.1%.

Prior to statistical analyses, data were $\log_{10}$-transformed. As data were assumed to be non-parametric, relative abundances of all 224 metabolites were compared between the exposed and unexposed workers using a Wilcoxon rank sum test. *P*-values were adjusted using the Benjamini-Hochberg method to control false discovery rates. In order to find a larger potential set of metabolites that could distinguish between exposure groups, a false discovery rate (FDR) ≤ 0.1 was considered significant.

For the metabolites found to be significantly differentially abundant between the exposed and unexposed groups, the exposure-response relationship was explored by using box-plots to visualize relative abundances in Mn exposed and unexposed groups. Data analysis was completed in R (version 3.6.1) and R Studio (version 1.2).

To identify pathways that may have been perturbed between individuals exposed and unexposed to Mn, a pathways analysis of the 224 metabolites was carried out using MetaboAnalyst 4.0 (22). This MetaboAnalyst module combines an enrichment analysis, which calculates whether groupings of metabolites in the same metabolic pathway differ significantly between exposure groups, and a pathway topology analysis, which assigns an impact score to each pathway. A higher impact score is indicative of not only more significant perturbations in the pathway, but also biologically meaningful changes in the measured metabolites (24).

To undertake the pathways analysis, the 224 metabolites identified from the targeted assay were matched to their Human Metabolome Database identifier for upload to MetaboAnalyst 4.0, where their relative abundances by exposure group were compared to the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolites pathway library for *Homo sapiens*. The Global Test option in MetaboAnalyst 4.0 was used to evaluate relative abundance differences among groups of metabolites in the same metabolic pathway, with these differences being used to calculate Benjamini-Hochberg FDRs between the exposed and unexposed groups. The betweenness centrality option (shortest path between nodes) was used to calculate metabolite importance (25, 26). With this method, the location of the metabolite in the pathway is considered, so when perturbed metabolites are central to the pathway and operate near each other or in succession to each other, it is assumed the pathway could be more impacted. If perturbed metabolites are only marginal to the pathway or relatively isolated compared to other perturbed metabolites in the pathway, then that pathway receives a lower impact score (27).

RESULTS

Table 1 shows participant demographics for the Mn-exposed and -unexposed workers included in this study. While both exposed and unexposed workers were predominantly White, there were more Hispanic workers in the Mn-exposed group. The Mn-exposed group was also slightly younger than the Mn-unexposed group. Nearly half of the Mn-unexposed workers were working on a night shift, whereas the Mn-exposed workers were predominantly first shift workers. No Mn-unexposed workers wore a respirator, but 10 of the Mn-exposed workers self-reported wearing an N95 or dust mask at least some of the time on the day their urine sample was collected. The lack of a formal respiratory protection program at the foundry and the observed poor respiratory hygiene allowed us to infer any mask use would have a limited effect on the received Mn dose.

Of the 224 metabolites identified from the targeted assay, seven were found to be significantly differentially abundant between groups defined by Mn exposure at an FDR $\leq$ 0.1: n-isobutyrylglycine, cholic acid, anserine, beta-alanine, methionine, n-isovalerylglycine, and threonine. These seven metabolites are outlined in **Table 2**, and boxplots showing their abundances in the exposure groups are shown in **Figure 1**. **Table 2** also provides information on their source, chemical class, and biological role in human metabolism. Results from Wilcoxon rank sum test for all 224 metabolites (including their adjusted *P*-values) are included in the **Supplementary Material**.

A pathways analysis of 80 *Homo sapiens* metabolic pathways was undertaken with the normalized data to determine if different metabolic pathways were perturbed between the

TABLE 1 | Participant demographics.

	Mn-exposed workers (*n* = 17) Mean ± SD (range)	**Mn-unexposed workers (*n* = 15) Mean ± SD (range)**
Age (at time of sample)	43.1 ± 12.0 (25, 66)	49.3 ± 10.1 (26, 60)
	***n* (%)**	***n* (%)**
Ethnicity		
Hispanic	9 (53)	4 (27)
Non-Hispanic	8 (47)	11 (73)
Race		
White	13 (76)	13 (87)
Non-White	4 (24)	2 (13)
Respirator		
Yes	10 (59)	0 (0)
No	7 (41)	15 (100)
Smoker		
Current	2 (13)	3 (18)
Previous	7 (47)	9 (53)
Never	6 (40)	5 (29)
Shift		
First	15 (88)	8 (53)
Second	2 (12)	7 (47)

TABLE 2 | Normalized, $\log_{10}$-transformed relative abundance of the seven metabolites found to be significantly differentially abundant (FDR $\leq$ 0.10) between Mn-exposed and -unexposed participants, and their biological details.

	Mn-exposed (*n* = 17)			**Mn-unexposed (*n* = 15)**						
Metabolite	**Mean (SD)**	**CV (%)**	**Detected %***	**Mean (SD)**	**CV (%)**	**Detected %***	**FDR****	**ESI**	**Chemical class**	**Source**	**Biological role**
n-Isobutyrylglycine (28)	5.68 (0.19)	3	100	5.40 (0.15)	3	100	0.02	Negative	Acylglycine	Endogenous	Urinary metabolite of fatty acids
Cholic acid (29)	3.43 (0.77)	22	41	4.41 (0.62)	14	87	0.03	Negative	Bile acid	Endogenous	Facilitates fat absorption and cholesterol excretion
Anserine (30)	6.25 (0.69)	0.1	100	5.42 (0.48)	9	100	0.03	Negative	Dipeptide	Endogenous	Biochemical buffer, chelator, antioxidant, and anti-glycation agent
Beta-alanine (31)	4.50 (0.98)	22	76	3.26 (0.84)	26	20	0.03	Positive	Beta amino acid	Endogenous	Aids in production of carnosine, which plays a role in muscle endurance
Methionine (32)	4.42 (0.19)	4	100	4.19 (0.15)	4	100	0.03	Positive	Amino acid	Exogenous	Important in growth of blood vessels, protein biosynthesis
n-Isovalerylglycine (33, 34)	6.33 (0.20)	3	100	6.10 (0.19)	3	100	0.08	Negative	Acylglycine	Endogenous	Urinary metabolite of fatty acids
Threonine (35)	6.03 (0.16)	3	100	5.86 (0.13)	2	100	0.10	Positive	Amino acid	Exogenous	Protein biosynthesis, deficiency related to neurological dysfunction

**Detected % refers to the number of samples in which this metabolite was detected.*

***Benjamini-Hochberg adjusted p-value between Mn-unexposed (n = 15) and Mn-exposed (n = 17) participants, from Wilcoxon rank-sum test.*

FDR, false discovery rate; SD, standard deviation; CV, coefficient of variation; ESI, electrospray ionization mode.

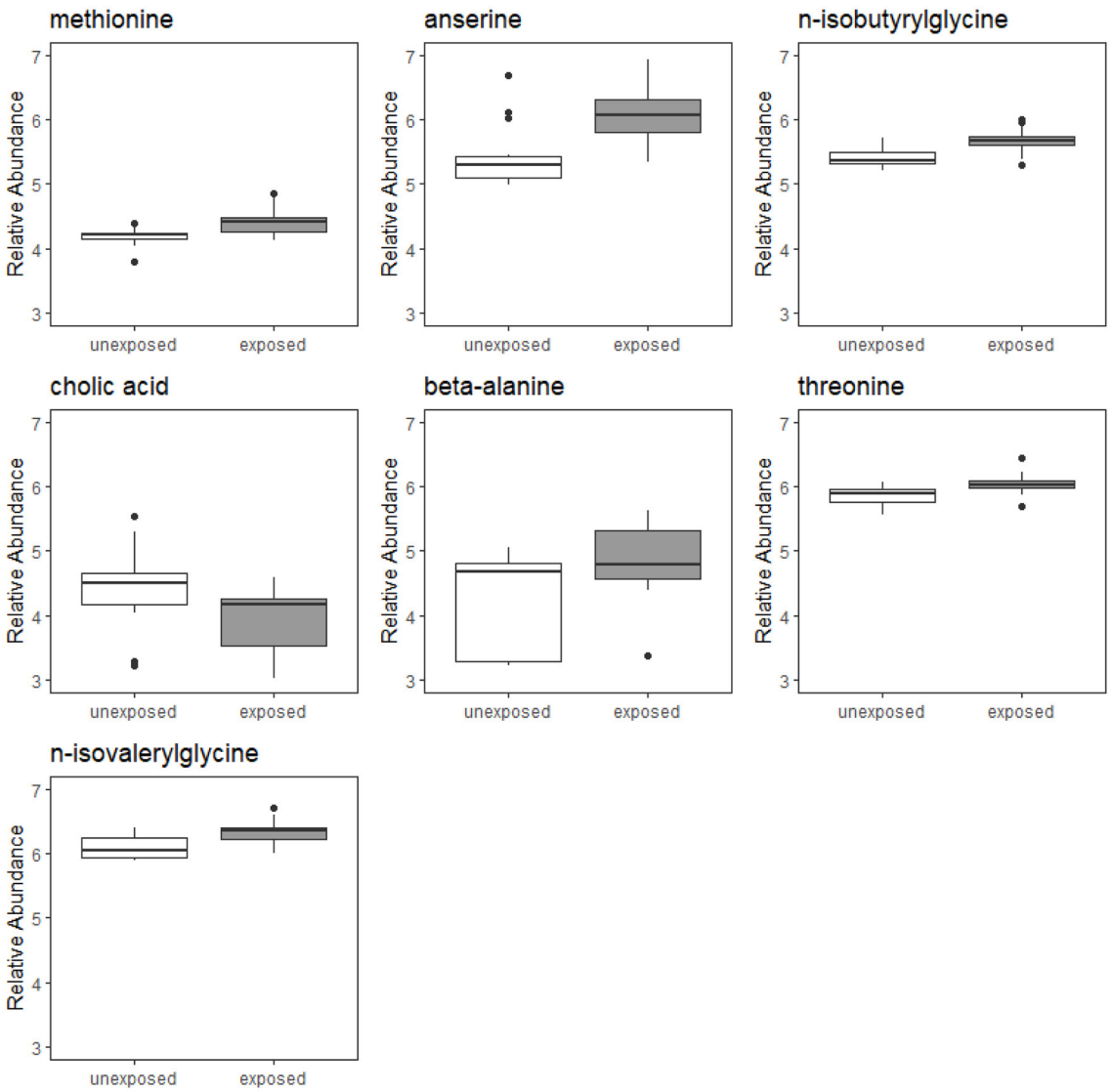

FIGURE 1 | Boxplots of relative abundances of seven metabolites found to significantly differentially abundant between Mn-exposed and -unexposed workers. For each exposure group, the middle line that divides the box into two parts represents the median value while the top and bottom lines of the box represent the 75th and 25th percentiles, respectively. The box represents the interquartile range (IQR) of scores for the group. The whiskers are extended to all values that are no >1.5 × IQR from the edge of the box.

Mn-unexposed and -exposed groups. Twenty-three pathways were identified that included at least four of the 224 metabolites, which is considered the minimum number for meaningful pathway analysis (36, 37). Of these 23 pathways, seven had a FDR $\leq$ 0.1, indicating a significant perturbation in the pathway between exposed and unexposed workers: beta-alanine metabolism, propanoate metabolism, pyrimidine metabolism, pantothenate and CoA biosynthesis, primary bile acid biosynthesis, histidine metabolism, and glycine, serine, and threonine metabolism. Of these significantly perturbed pathways, three had an impact score >0.5, indicating the perturbed metabolites in that pathway were at least moderately central to the pathway and operate near or in succession to each other: beta-alanine metabolism, histidine metabolism, and glycine, serine, and threonine metabolism. **Table 3** outlines the pathways that were found to be significantly perturbed between exposed and unexposed workers, including the impact scores for each of these pathways.

DISCUSSION

Here, differences in 224 metabolites measured using a targeted metabolomics LC-MS/MS platform were explored between Mn-exposed and -unexposed workers. Seven metabolites were found to be significantly differentially abundant between exposure groups. When investigating which pathways were perturbed in

TABLE 3 | Pathway enrichment analysis.

Pathway	Compounds in pathway*	Compounds in data*	FDR**	Impact
Beta-alanine metabolism	21	7	0.003	0.56
Propanoate metabolism	23	5	0.01	0.04
Pyrimidine metabolism	39	8	0.01	0.19
Pantothenate and CoA biosynthesis	19	6	0.01	0.06
Primary bile acid biosynthesis	46	6	0.01	0.04
Histidine metabolism	16	7	0.05	0.64
Glycine, serine, and threonine metabolism	33	16	0.08	0.66

**Compounds in pathway refers to the total number of compounds operating in that pathway, including compounds that were not investigated in our dataset; Compounds in data refers to the number of compounds in the pathway that were in our data set of 224 metabolites.*

*** Benjamini-Hochberg adjusted p-value characterizing the differences in the pathways between Mn unexposed (n = 15) and Mn exposed (n = 17) participants.*

FDR, false discovery rate.

exposed workers as compared to unexposed workers, pathways related to amino acid metabolism (beta-alanine metabolism, histidine metabolism, and glycine, serine, and threonine metabolism) were significantly perturbed in the Mn-exposed group and had the highest impact scores.

While differences in metabolite abundance between Mn-exposed and -unexposed workers have not previously been investigated using a targeted metabolomics platform, others have examined differences in metabolomics profiles between workers exposed and unexposed to welding fume, which typically includes high levels of Mn. In a study of 52 boilermakers, Shen et al. (38) utilized an untargeted metabolomics approach to look at changes in plasma collected pre- and post-welding shift. These untargeted data were generated using a mass spectrometry platform. The authors found that the metabolic changes over the work shift were related to changes in lipid pathways and amino acid utilization, both of which are associated with inflammation. Wang et al. (34) compared the urine metabolomics profile of 10 welders and 6 office workers using untargeted data generated from a nuclear magnetic resonance (NMR) platform. After identifying the NMR bins found to be significantly different between groups, the authors found higher levels of several amino acids, creatinine, and acetone among welders, and lower levels of creatine. The authors hypothesized many compounds found to be higher in welders are important in modulating inflammatory and oxidative tissue injury processes. Notably, to control for some potential confounding, Wang et al. only included participants who did not smoke cigarettes or drink alcohol and took urine samples after overnight fasting, whereas similar steps were not taken in the study presented here.

Despite representing three different cohorts exposed to Mn-containing fumes and metabolomics data generated from three different analysis platforms, Shen et al., Wang et al., and the work presented here all found amino acid perturbations in groups exposed to Mn-containing fumes. Further research is needed to understand the potential importance of amino acids in the Mn exposure-disease continuum, and whether elevated levels of particular amino acids are consistently related to exposure to Mn.

For exposure studies utilizing metabolomics, it can be challenging to determine if the differences between groups are truly due to the measured exposure, or if unmeasured co-exposures in the workplace may be driving the observed differences. Foundry work could have a number of co-exposures that differ from those encountered by crane operators/truck drivers at a metal recycling center. These include substantial exposure to silica and carbon monoxide, in addition to polycyclic aromatic hydrocarbons (PAHSs), phenol, formaldehyde, isocyanates, and amines among foundry workers. Foundry workers could also have exposures to other metals such as chromium, nickel, and iron. In this study, co-exposures were not assessed due to operational constraints. Metabolites are also subject to a variety of internal and external cues, and bodily concentrations dynamically change throughout the day due to circadian rhythms, activities the person is performing, food the person is eating, and other constantly occurring internal biological processes. For example, two of these seven metabolites of interest to this study are essential amino acids, which likely differ between the groups due to differences in diet as that is their predominant source.

Here, urine samples were collected from each subject at the end of their work shift, but this would represent different clock times for workers on a first shift and second shift, which could influence metabolite levels. However, when stratifying the seven metabolites presented in **Table 2** by shift for unexposed workers, no differences in distributions were seen, though power to detect differences was limited. While it is impossible to control all sources of within- and between-person variability in an occupational setting, care should be taken to ensure samples are taken at the same time of day. Appropriately-timed repeat samples can also be informative for understanding the variability in changes to metabolites related to exposure.

Similarly, co-variates were not collected on biological and behavioral characteristics that can influence metabolomics, including body mass index (BMI), pre-existing conditions, dietary habits, or use of pharmaceutical agents. Unfortunately, the lack of repeat measures, lack of information on co-exposures, and lack of co-variates collected must be acknowledged as a major limitation in this study and something that should be accounted for in future occupational metabolomics studies.

Additionally, we did not undertake any validation of study findings to see if the metabolites found to be significantly differentially abundant in these exposed workers remained so in other Mn-exposed workers, or in a testing set of samples from this cohort. This would be an important

step to increase the external validity of this study, and to further confirm the biological relevance of the findings. Future occupational metabolomics studies should strive to enroll sufficient participants to split their data into separate training and testing sets, or utilized data from an external dataset for testing and validation purposes. The sample size of this study, which was originally conceived of as a pilot study, was a major contributor to the lack of power to detect differences between the 224 compounds investigated here.

The potential value of targeted metabolomics for occupational exposure studies must also be noted. Targeted metabolomics allows the occupational health researcher to investigate a range of known metabolites that may relate to different exposures the worker has experienced, both at work and in other environments. This makes metabolomics an important tool for characterizing the exposome, which refers to the totality of exposures that someone has encountered throughout their lifetime (39). Occupational settings are a particularly valuable place to develop metabolomics methods for exposure assessment, given the higher exposures typically experienced in workplace environments and the prevalence of otherwise uncommon exposures in workplace environments. With work schedules following consistent patterns, it can also help with ensuring consistency in sample collection.

In conclusion, this work continued to explore the utility of metabolomics for distinguishing between groups defined by occupational exposures. Findings from this study were consistent with other studies of workers exposed to Mn-containing fumes, showing perturbations of amino acids and amino acid pathways. While further study is warranted to explore the potential role of amino acids in Mn toxicity, we hope that the work presented here encourages others to integrate targeted metabolomics into their human exposure studies, in order to continue to expand the use of this promising technique as a means of hypothesis generation and biomarker discovery in occupational health and exposome studies. We also hope that the limitations we outlined here will ensure that subsequent occupational health researchers can collect more rigorous co-variates with their biosamples to better inform results from planned or subsequent metabolomics analyses.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: github.com/bakermarissa/targeted_mn_metabolomics.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Washington IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MGB and CDS conceived of and designed the original research study. MGB completed all data collection and secured funding for analyses presented here. DR oversaw all laboratory analyses. MGB, KAC, and DR undertook data analysis and interpretation. KAC and MGB drafted the article. KAC, MGB, CDS, and DR undertook critical revision of article. All authors gave final approval of the version to be published and as such all agree to be held accountable for the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.666787/full#supplementary-material

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Micronucleus Formation Induced by Glyphosate and Glyphosate-Based Herbicides in Human Peripheral White Blood Cells

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Glyphosate is the most commonly used herbicide around the world, which led to its accumulation in the environment and consequent ubiquitous human exposure. Glyphosate is marketed in numerous glyphosate-based herbicide formulations (GBHs) that include co-formulants to enhance herbicidal effect of the active ingredient, but are declared as inert substances. However, these other ingredients can have biologic activity on their own and may interact with the glyphosate in synergistic toxicity. In this study, we focused to compare the cytogenetic effect of the active ingredient glyphosate and three marketed GBHs (Roundup Mega, Fozat 480, and Glyfos) by investigating cytotoxicity with fluorescent co-labeling and WST-1 cell viability assay as well as genotoxicity with cytokinesis block micronucleus assay in isolated human mononuclear white blood cells. Glyphosate had no notable cytotoxic activity over the tested concentration range (0–10,000 μM), whereas all the selected GBHs induced significant cell death from 1,000 μM regardless of metabolic activation (S9). Micronucleus (MN) formation induced by glyphosate and its formulations at sub-cytotoxic concentrations (0–100 μM) exhibited a diverse pattern. Glyphosate caused statistically significant increase of MN frequency at the highest concentration (100 μM) after 20-h exposure. Contrarily, Roundup Mega exerted a significant genotoxic effect at 100 μM both after 4- and 20-h exposures; moreover, Glyfos and Fozat 480 also resulted in a statistically significant increase of MN frequency from the concentration of 10 μM after 4-h and 20-h treatment, respectively. The presence of S9 had no effect on MN formation induced by either glyphosate or GBHs. The differences observed in the cytotoxic and genotoxic pattern between the active principle and formulations confirm the previous concept that the presence of co-formulants in the formulations or the interaction of them with the active ingredient is responsible for the increased toxicity of herbicide products, and draw attention to the fact that GBHs are still currently in use, the toxicity of which rivals that of POEA-containing formulations (e.g., Glyfos) already banned in Europe. Hence, it is advisable to subject them to further comprehensive toxicological screening to assess the true health risks of exposed individuals, and to reconsider their free availability to any users.

Keywords: glyphosate, formulation, GBHs, micronucleus, cytotoxicity, genotoxicity

INTRODUCTION

Our consumer society has reached the point where a chemical marketed in the United States in the 1970s and declared harmless for decades is virtually everywhere: in natural waters, meat, wine, beer, and even in the urine of many of us (1, 2). This chemical is one of the pesticides with the highest sales volume on the market, glyphosate. It is the active ingredient of numerous glyphosate-based herbicides (GBHs), and due to its use in small gardens, it is perhaps the best-known agrochemical. To date, there is no substitute that would produce the same efficacy as glyphosate, and we have to expect a large rate of crop losses worldwide if it is ever banned. Nevertheless, there is a real civil rights movement against glyphosate in the United States today, in the courts, because the International Agency for Research on Cancer (IARC) classified glyphosate as a "probable human carcinogen" in March 2015 (3). As a result, by the end of 2019, more than 40,000 lawsuits had been filed by American citizens suffering from non-Hodgkin lymphoma (NHL) and presumably exposed to glyphosate-based herbicides, three of whom have gone to trial and each won the lawsuit (4). At the same time, surprising developments have come to light. Neither the European Food Safety Authority (EFSA) nor the US Environmental Protection Agency (EPA) did find sufficient evidence about the carcinogenic potential of the herbicide in October 2015 and September 2016, respectively (5, 6). The discrepancy between the findings of each agency was attributed to the facts that EFSA and EPA relied mostly on unpublished studies funded by herbicide manufacturers, while IARC did not. In addition, IARC placed heavy weight on the cocktail effect of formulated GBHs (i.e., when glyphosate is used with another chemical in a formulation) whereas EFSA and EPA did not (4, 7). In October 2017, the European Parliament supported the withdrawal of glyphosate within 5 years, but 2 months later the European Commission voted to re-authorize the active substance in the EU for 5 more years (8). This decision raises deep concern in the light of the findings of recent meta-analytic studies that, combining results of numerous epidemiological investigations, have identified a compelling link between real-life glyphosate exposure and NHL (9, 10).

In reality, almost no one is exposed to glyphosate as an active substance alone, but rather to complete GBHs, which contain various other ingredients labeled as "adjuvants" or "co-formulants" that are aimed to improve the herbicidal efficacy of glyphosate, but are defined as inerts (11). Usually, the identity and concentration of supposedly inert co-formulants in GBHs are not disclosed on product labels, material and safety data sheets, or in any publicly accessible documentation of the pesticide products because they constitute business secrets (12). Therefore, the composition of numerous GBHs is unknown, that makes it very difficult for scientists to assess the health risks of certain adjuvants or the combined effects of different ingredients in GBHs. Notwithstanding, more and more evidence has emerged that certain adjuvants, such as polyethoxylated tallow amines (POEAs), are more toxic than glyphosate alone (13, 14), and/or increase the toxicity of the active substance by allowing it to penetrate plant, but also animal and human, cells more easily, increasing formulations' toxicity (15, 16). As a result of this recognition, GBHs containing these adjuvants are progressively being phased out and replaced by a new generation of co-formulants on the European market, but not in the US. The toxic potential of GBHs formulated with non-POEA adjuvants is reported to be lower than that with POEAs (17); nevertheless, there is still a considerable knowledge gap in the systematic assessment of chronic health risks posed by the exposure to commercially available GBHs with still highly variable composition.

The IARC classification for glyphosate was partly based on strong evidence that glyphosate or GBHs are able to induce genotoxicity, recognized first step in carcinogenesis, in human cells *in vitro* and in experimental animals (3). Recently, comprehensive reviews summarizing animal carcinogenicity data and data from multiple *in vitro* and *in vivo* genotoxicity assays have further supported the IARC statement (18, 19). DNA damage indicated by DNA strand breaks as a marker of genotoxicity was observed as a result of exposure to glyphosate as an active ingredient in various human cell types *in vitro* (20–25) as well as in mice (26) and fish (23, 27, 28) *in vivo*. GBHs induced DNA damage in human liver HepG2 cells (29), buccal carcinoma cells (TR146) (21) and peripheral blood mononuclear cells (30) *in vitro* as well as in mice *in vivo* (26). Another marker which can help to assess the genotoxic properties of xenobiotics is the presence of micronuclei (MN) that indicates clastogenic events in eukaryotic cells. MN can be formed from acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division, and can be detected by the cytokinesis-block micronucleus (CBMN) assay, which is a standardized, sensitive and simple laboratory technique to evaluate genomic damage in isolated cells (31). MN was successfully detected as a biomarker in several human biomonitoring studies of pesticide-exposed individuals (32–34) including GBH-exposed humans (35). A large-scale systematic meta-analytical review involving 93, mainly non-human, experimental studies has been recently carried out to analyze the relationship between exposure to glyphosate or GBHs and the formation of MN. The review concluded in general that both the active ingredient and the formulations increase the frequency of MN in tested organisms (36). Examples include, but are not limited to, studies reporting MN formation after exposure to glyphosate as an active ingredient in isolated human lymphocytes (22, 37) and HepG2 cells (38) *in vitro* and in mice (26) *in vivo*. GBHs were also able to induce MN formation in polychromatic erythrocytes of mice (26, 39) *in vivo*.

Although genotoxic and mutagenic effects of glyphosate and GBHs have already been extensively studied with various methods, there is still insufficient evidence on the possible complex interactions between ingredients in GBHs, and the effect of glyphosate-based formulations on the induction of MN formation in human cells has not been investigated so far. As a continuation of our previous study that focused on the comparative analysis of primary DNA damage induced by three marketed GBHs with different composition and the active ingredient glyphosate, herein, we compare the clastogenic activity of the same GBHs to glyphosate as well as to each other in

human mononuclear white blood cells (HMWB) *in vitro* using the CBMN assay.

MATERIALS AND METHODS

Chemicals

Analytical-grade glyphosate (N-(phosphonomethyl) glycine, CAS No: 1071-83-6) was purchased from VWR International Kft (Debrecen, Hungary). Samples of three GBHs, namely

- Roundup Mega containing 551 g/L or 42% (w/w) potassium salt of glyphosate (CAS No: 70901-12-1; equivalent to 450 g/L glyphosate) and 7% (w/w) ethoxylated etheralkylamine (CAS No: 68478-96-6);
- Fozat 480 containing 480 g/L or 41% (w/w) isopropylammonium salt of glyphosate (CAS No: 38641-94-0; equivalent to 360 g/L glyphosate) and <5% (w/w) hygroscopic substances;
- Glyfos containing 480 g/L or 42% (w/w) isopropylammonium salt of glyphosate (equivalent to 360 g/L glyphosate) and 9% (w/w) polyethoxylated tallow amine (CAS No: 61791-26-2);

were kindly provided by pesticide applicators. Composition data for each formulation were retrieved from the material safety data sheets (MSDS). Chemicals used for the assays and human liver-derived metabolic activation system (S9 fraction) were obtained from Sigma-Aldrich Chemie GmbH (Heidelberg, Germany). Cell culture medium and its supplements were obtained from Biowest (Nuaillè, France). The acetomethoxy derivative of calcein (Calcein AM) and propidium iodide (PIO) fluorescent dyes were purchased from Biotium (Hayward, CA, USA). Heparin-containing vacutainers were purchased from BD Vacutainer Systems (Plymouth, UK).

Cell Cultures

Human peripheral whole blood samples were obtained by venipuncture and collected into heparin-containing vacutainer tubes from three non-smoking, healthy volunteers (males, aged 20–40 years) without known previous contact with pesticides. Cultures were prepared within 1-h of phlebotomy. 0.3 mL heparinized whole blood was added to 4.7 mL RPMI-1640 complete medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL amphotericin and 1.5% phytohemagglutinin. Whole blood samples were cultured for 48-h before treatment. All donors signed the informed consent. The study was approved by the Hungarian Ethical Committee for Medical Research (document 147-5/2019/EÜIG) and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki.

Cell Treatment

The cells were exposed to glyphosate at final concentrations of 0.1, 1, 10, 100, 1,000, 10,000 μM and to three GBHs at the same glyphosate-equivalent final concentrations. The concentrations of GBHs are referred to as glyphosate equivalent concentrations in this study.

The stock solutions and the dilution series were made in phosphate-buffered saline (PBS) and adjusted to pH 7.2. Aliquots of different concentrations of glyphosate and GBH solutions, as well as PBS as negative control and 1.3 μM bleomycin sulfate (BLEO) as a positive control, were added to the cell cultures and incubated for 4- and 20-h at 37°C. The PBS content was always <10% (v/v) in the cell culture medium.

All the experiments were conducted in the presence and absence of S9 fraction. Hundred microliter of the working S9 mix containing 10% (v/v) of S9 fraction was composed of 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP was added to the S9+ samples.

Calcein AM and Propidium Iodide Cell Viability Assay

After treatment and removal of erythrocytes by hypotonic (0.075 M KCl) lysis, Calcein AM and PIO fluorescent dyes were used to co-label the HMWB cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. PIO is a DNA intercalating dye, which is able to permeate membranes of dead and dying cells but cannot penetrate plasma membranes of live healthy cells.

Both fluorescent dyes were dissolved in PBS (pH 7.2) to a final concentration of 2 μM each. Two hundred microliter of this working solution were added to the cell pellets (1×10^5 cells) and incubated for 30 min at 4°C, protected from light. The labeled cells were washed and re-suspended in ice-cold PBS buffer. Forty microliter of the cell suspension was put on a microscope slide for immediate microscopic examination.

FITC filter for Calcein AM and TRITC filter for PIO was applied to excite the co-labeled cells. Survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10 to 30 images.

WST-1 Cell Viability Assay

The WST-1 cell proliferation reagent was applied according to the manufacturer's protocol. Before treatment, HMWB cells were separated from erythrocytes by density-gradient centrifugation over Histopaque-1077 gradient to avoid interference caused by residual hemoglobin during absorbance measurement that would have resulted from using hypotonic lysis of erythrocytes. The buffy coat was then aspirated and re-suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). HMWB cells were seeded in Eppendorf tubes at a cell number of 1×10^5 and were treated with the test chemicals as described in the Cell treatment section. Following treatment, samples were centrifuged, the supernatant was discarded, and HMWB cells were resuspended in 100 μL RPMI-1640 complete medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin. Samples were then transferred to flat-bottomed 96-well plate, and 10 μL of WST-1 was added directly to the culture in each well. The cells were incubated for 3-h at 37°C. Absorbance at 440 nm was measured using an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). The reference absorbance was set at 700 nm. Cell viability was calculated by

dividing the absorbance of the treated cells by that of the vehicle-treated (PBS) control cells (considered 100%).

Cytokinesis-Block Micronucleus Assay

CBMN assay was carried out following the previously reported standardized protocol (OECD guideline) (40) with slight modifications (41). After 4-h treatment with glyphosate alone and with the three GBHs, whole blood cells were centrifuged, the supernatant was removed, and the cells were resuspended in 3 μg/mL cytochalasin B containing medium. In case of 20-h treatment, cytochalasin B was added in parallel with the addition of the test chemicals. Following 20-h incubation, cells were harvested for slide preparation. Whole blood samples were centrifuged and resuspended in hypotonic (0.075 M) KCl solution then fixed in cold fixative (methanol:acetic acid 5:1) for 30 min at room temperature. The latter step was repeated twice to completely remove the erythrocytes.

Cell suspensions were carefully dropped onto clean wet slides to disperse the cells. Slides were air dried, stained with 3% Giemsa in distilled water and mounted in Eukitt.

Giemsa-stained slides were coded and analyzed blindly by two scorers under a magnification of 400. Proliferation index (PI) was determined by counting at least 500 cells with one, two or more than two nuclei. The PI was calculated according to the formula: $PI = M_1 + 2M_2 + 3M_{multi}/n$, where M_1 to M_{multi} represent the number of cells with one to multiple (more than 2) nuclei and n is the number of cells scored. In total, 2,000 binucleated cells (1,000 per slide) were scored from each experimental point. The MN frequency was calculated as the ratio of the number of binucleated cells with micronuclei (BNMN) to the binucleated cells. The identification of MN was performed according to the criteria described by Fenech et al. (42).

Data Analysis

Experiments were independently performed three times from three different donors. Cell viability was expressed as the mean proportions of living cells from repeated experiments. The rate of cell viability, the frequency of binucleated cells with micronuclei and the proliferation index induced by various concentrations of the test chemicals in repeated experiments were statistically compared to that of untreated cells using ANOVA with Dunnett's *post hoc* test. The same statistical test was used to analyze the effect of metabolic activation by comparing the micronucleus frequency and proliferation index of S9-treated and S9-untreated samples at each exposure concentration. Statistically significant difference was accepted at 5% significance level.

RESULTS

Cell Viability

The viability of HMWB cells treated with glyphosate alone for 4- and 20-h was found to be over 80% in the absence and presence of S9 over the entire concentration range in both cell viability assays (**Figures 1**, **2**). A slight but statistically significant decrease of cell viability could be noticed only at 10,000 μM without S9 treatment in the fluorescent co-labeling assay at 4- and 20-h exposure, too. In contrast to the active ingredient, all the three GBHs induced a significant decrease in the proportion of living cells from 1,000 μM regardless of metabolic activation, which was more evident when using WST-1 cell viability assay. It detected significantly higher cell death at 1,000 μM concentration of the formulations compared to the fluorescent co-labeling assay.

Micronucleus Induction

In line with the OECD Test Guideline for the *in vitro* micronucleus assay suggesting that concentrations that induce cytotoxicity >55 ± 5% should be excluded from genotoxicity testing (40), CBMN assays were carried out in a previously determined, sub-cytotoxic concentration range of the tested compounds, because cytotoxic processes, especially apoptosis, could potentially act as confounders in genotoxicity assays. As GBHs from 1,000 μM were able to be induce statistically confirmed cell death in the cytotoxicity assays, only concentrations of 0.1, 1, 10, and 100 μM were investigated in the CBMN assay.

Glyphosate alone did not cause a statistically significant increase of MN frequency except at the highest concentration (100 μM) after 20-h exposure in the absence (8.69% ± 2.34%, $p < 0.01$) and presence (9.49% ± 1.07%, $p < 0.001$) of S9, as well. By contrast, all the three GBHs induced significant increase of MN frequency at 100 μM both after 4-h with (Roundup Mega: 9.11% ± 2.76%, $p < 0.05$; Fozat 480: 14.50% ± 4.84%, $p < 0.05$; Glyfos: 9.11% ± 1.69%, $p < 0.01$) and without (Roundup Mega: 9.67% ± 1.54%, $p < 0.05$; Fozat 480: 13.93% ± 3.27%, $p < 0.01$; Glyfos: 10.01% ± 1.67%, $p < 0.05$) metabolic activation, as well as after 20-h in the absence (Roundup Mega: 10.12% ± 1.98%, $p < 0.05$; Fozat 480: 10.43% ± 0.59%, $p < 0.001$; Glyfos: 10.30% ± 2.92%, $p < 0.01$) and presence (Roundup Mega: 7.62% ± 2.31%, $p < 0.05$; Fozat 480: 10.00% ± 1.81%, $p < 0.05$; Glyfos: 9.87% ± 3.04%, $p < 0.05$) of S9. Moreover, 4-h treatment with Glyfos (without S9: 6.52% ± 1.22%, $p < 0.05$; with S9: 7.72% ± 1.73%, $p < 0.05$) and 20-h treatment with Fozat 480 (without S9: 9.13% ± 1.52%, $p < 0.01$; with S9: 8.59% ± 2.11%, $p < 0.05$) at the concentration of 10 μM also resulted in a significant increase of binucleated cells with micronuclei (**Figure 3**, **Supplementary Table 1**).

The presence of metabolic enzymes did not significantly alter MN frequency induced either by glyphosate alone or by the GBHs.

The proliferation index did not show statistically significant changes with increasing concentrations of the test chemicals at both exposure times, regardless of the presence of the metabolic enzyme system (**Figure 4**, **Supplementary Table 2**).

DISCUSSION

This work is the first study investigating the MN inducing ability of three GBHs along with glyphosate in isolated human cells *in vitro*. To examine whether potential metabolites of the selected herbicides can cause damage to cells, human liver-derived metabolic enzyme system (S9) was also applied. Two of the examined GBHs, Roundup Mega and Fozat 480, are permitted in Hungary and in the EU, while the POEA-containing Glyfos was withdrawn from the Hungarian market in 2017 (43).

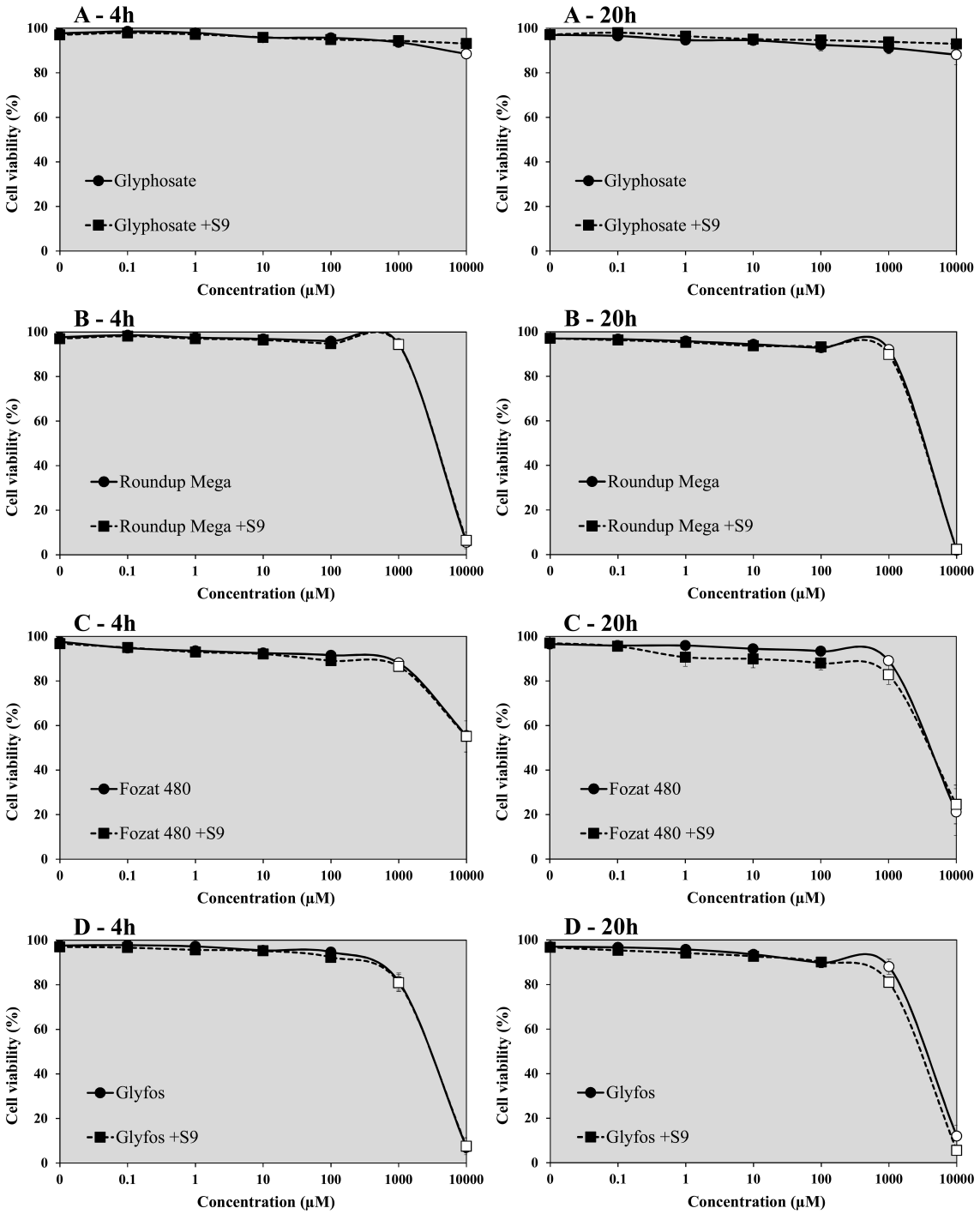

FIGURE 1 | Effect of 4- and 20-h exposure to increasing concentrations of glyphosate **(A)**, Roundup Mega **(B)**, Fozat 480 **(C)**, and Glyfos **(D)** on cell viability in the absence and presence (+S9) of metabolic activation system detected by fluorescent co-labeling. The data points indicate the means ± standard error of the mean (SEM) of three repeated experiments. Statistically significant decrease of cell viability, indicated by empty data points, was determined by comparing the values induced by various doses of glyphosate or GBHs to the background level of untreated cells by ANOVA with Dunnett's *post hoc* test.

Our data indicate that glyphosate alone could not considerably decrease the viability of HMWB cells up to 10,000 μM both after 4- and 20-h exposure regardless of metabolic activation, which is in line with our previous observations (44), and also with findings of Wozniak (30), but contradicts results of De Almeida's study, in which pure glyphosate induced a significant reduction in cell

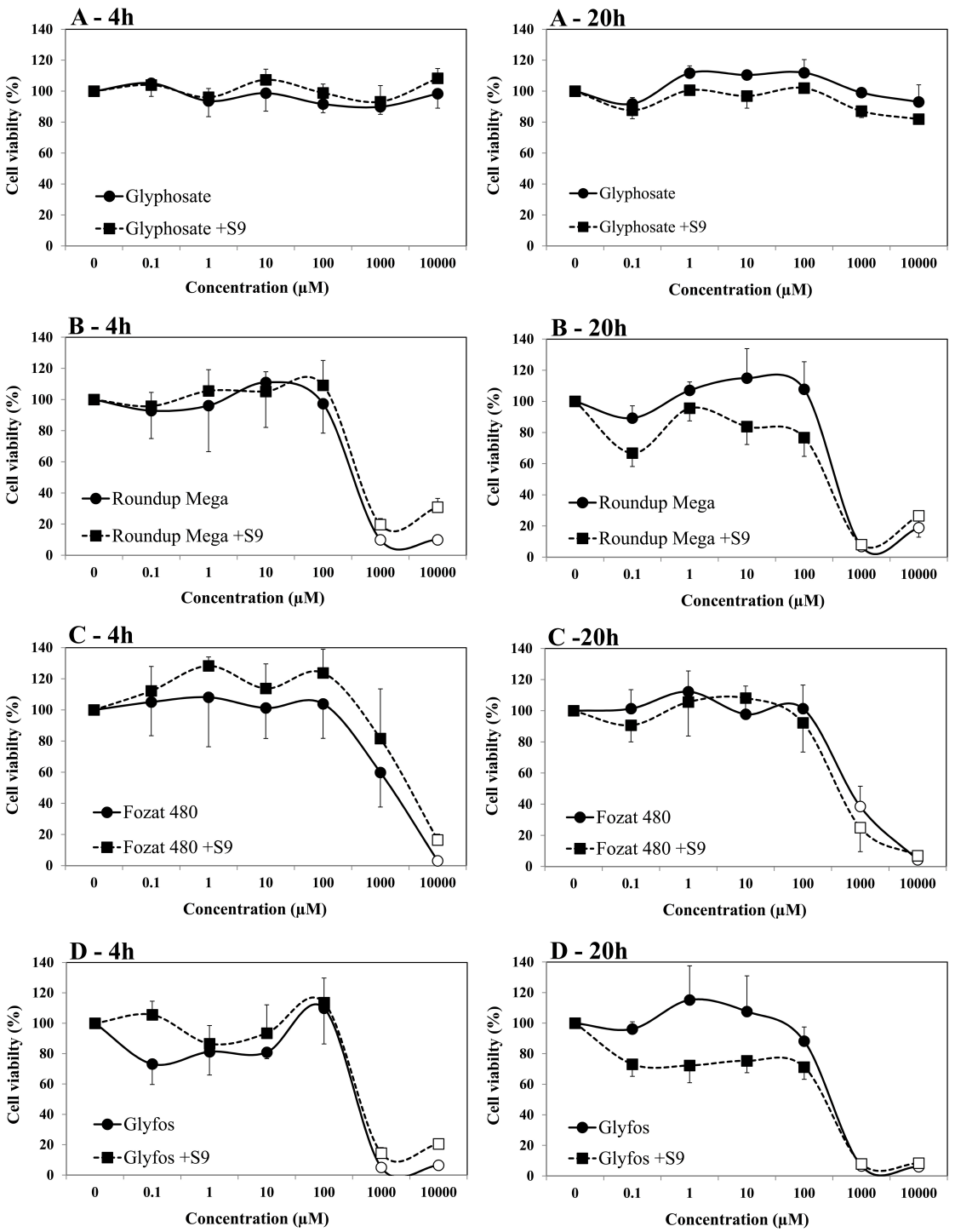

FIGURE 2 | Effect of 4- and 20-h exposure to increasing concentrations of glyphosate **(A)**, Roundup Mega **(B)**, Fozat 480 **(C)**, and Glyfos **(D)** on cell viability in the absence and presence (+S9) of metabolic activation system detected by WST-1 cell viability assay. The data points indicate the means ± standard error of the mean (SEM) of three repeated experiments. Statistically significant decrease of cell viability, indicated by empty data points, was determined by comparing the values induced by various doses of glyphosate or GBHs to the background level of untreated cells by ANOVA with Dunnett's *post hoc* test.

viability of whole blood from the concentration of 10 μg/mL (59.17 μM) as measured by the tetrazolium-based colorimetric (MTT) assay (45). This discrepancy may be attributed to the different cell types used (isolated HMWB cells vs. whole blood culture), therefore the comparability of results is limited. Unlike glyphosate, all the three GBHs showed pronounced

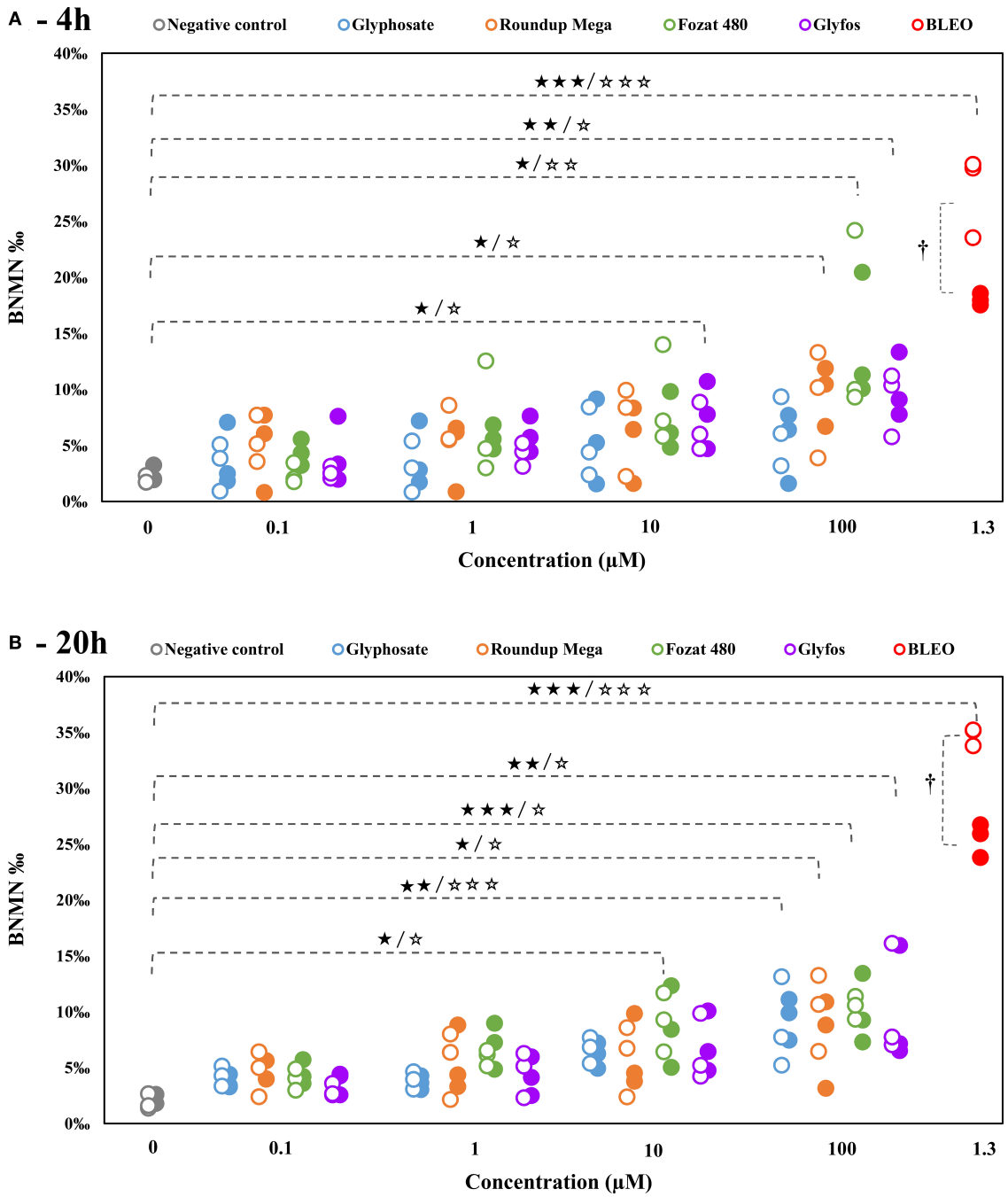

FIGURE 3 | Frequency of binucleated cells with micronuclei (BNMN %) induced by 4-h **(A)** and 20-h **(B)** exposure to sub-cytotoxic concentrations of glyphosate and GBHs without (○) and with (•) metabolic activation (S9) in human mononuclear white blood cells, detected by cytokinesis-block micronucleus assay. Data points are results of individual experiments. Statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) increase was determined by comparing the frequency of binucleated cells with micronuclei induced by various doses of test chemicals to the background level of untreated cells by ANOVA with Dunnett's *post hoc* test. The same test was used to detect statistically significant ($^{\dagger}p < 0.05$) difference in the frequency of binucleated cells with micronuclei between S9-treated and S9-untreated cells induced by the same concentration of test chemicals.

cell-killing activity at a high concentration range of 1,000 to 10,000 µM, especially in the WST-1 cell viability assay, regardless of the presence of the metabolic enzyme system and exposure durations. The increased cytotoxic potential of GBHs compared to pure glyphosate has been well-established and attributed to the presence of adjuvants in the formulations (13, 17, 29, 46–49).

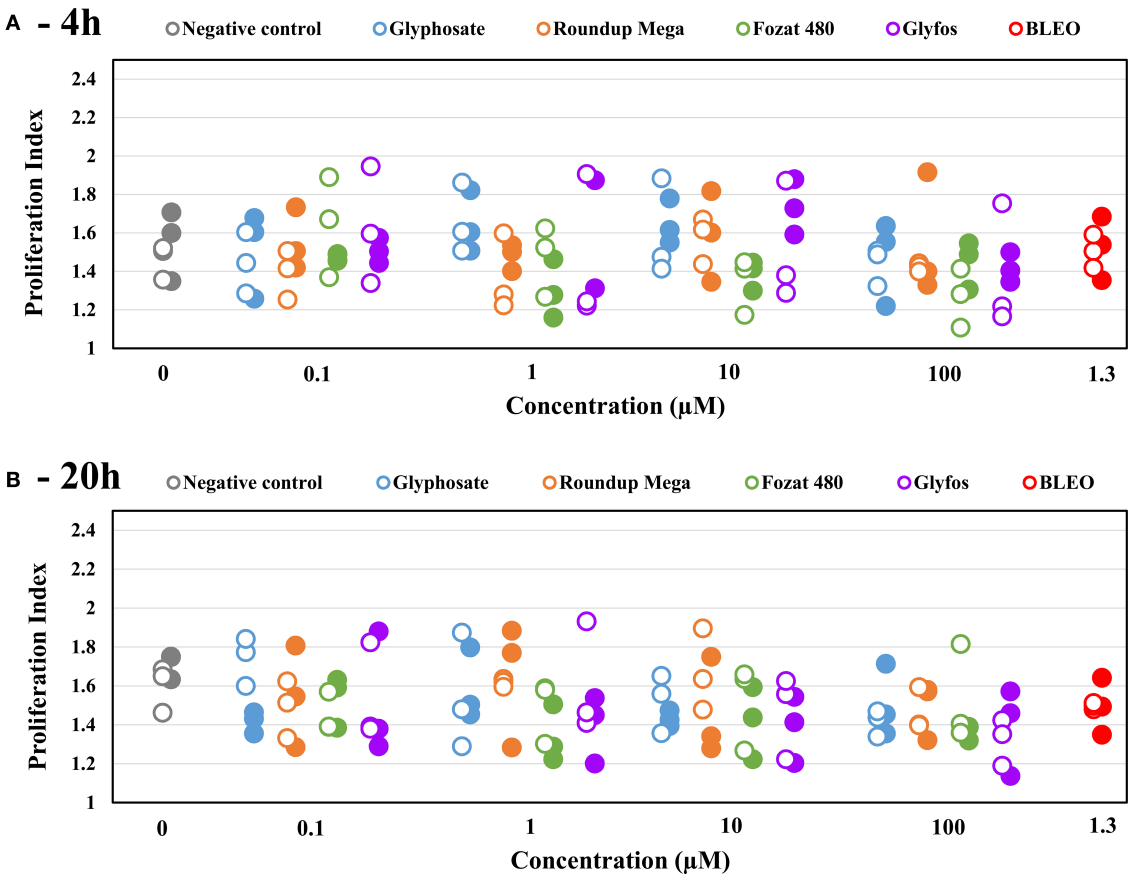

FIGURE 4 | Proliferation index (PI) induced by 4-h **(A)** and 20-h **(B)** exposure to sub-cytotoxic concentrations of glyphosate and GBHs without (○) and with (•) metabolic activation (S9) in human mononuclear white blood cells, detected by cytokinesis-block micronucleus assay. Data points are results of individual experiments. Statistically significant change was determined by comparing the proliferation index induced by various doses of test chemicals to the background level of untreated cells by ANOVA with Dunnett's *post hoc* test. The same test was used to detect statistically significant difference in the proliferation index between S9-treated and S9-untreated cells induced by the same concentration of test chemicals.

POEAs, the declared co-formulants in Glyfos, are reported to be over 1,000 times more cytotoxic than glyphosate alone (48, 49). Surprisingly, the different adjuvant content of the GBHs tested in this study did not considerably alter their cytotoxic effect, although the extent of cell death induced by Fozat 480 was less than that caused by the other two formulations, which can be explained by the lower concentration or toxicity of the other ingredients in Fozat 480. The adjuvant content of Fozat 480 (<5% hygroscopic substances) is not declared exactly in its MSDS; however, the dose-response relationship of cell viability suggests that it may also contain ethoxylated tallow amine surfactants, even if in lower concentration than the other two GBHs. This idea is supported by a study in which Fozat 480 exhibited a cytotoxicity pattern similar to other GBHs containing POEAs or ethoxylated ether alkylamine adjuvants (50). It is proven that ethoxylated adjuvants can disrupt cell membrane integrity and permeability, consequently increasing the bioavailability of glyphosate (51), but it is still not clear whether surfactants themselves are responsible for the cytotoxic effects or they interact with glyphosate synergistically. According to Wozniak et al. (30), genotoxicity detected in HMWB cells after exposure to technical glyphosate, Roundup 360 PLUS or the metabolite of glyphosate (aminomethylphosphonic acid, AMPA) was not the result of direct interaction of these compounds with the genetic material because no DNA adducts have been formed, rather due to effects mediated by reactive oxygen species (ROS) induced by the chemicals, which may also explain increased cell death. There is no clear evidence on the ROS-inducing potential of glyphosate (22, 25, 52); however, GBHs have been shown to induce oxidative stress (46, 53, 54), supporting the role of co-formulants in the cytotoxic effects observed in our experiments.

To avoid interference of cell death mechanism with genotoxic insults in our study, MN-inducing ability of the selected herbicides was investigated in a sub-cytotoxic (0–100 µM equivalent to glyphosate) concentration range. Technical glyphosate was able to produce a statistically significant increase of MN frequency in HMWB cells at the highest concentration of 100 µM after 20-h exposure in the absence and presence of S9. The observed lack of genotoxic effect in HMWB cells at lower concentrations and shorter exposure duration is in agreement

with findings reported by Mladinic et al., who also did not observe a significant increase in the proportion of micronuclei in human lymphocytes in the same concentration range (22). By contrast, Santovito et al. found MN-inducing effect of glyphosate in human lymphocytes in a much lower concentration range of 0.0125–0.5 μg/mL (0.07–2.9 μM), but after a 48-h incubation period (37). Low glyphosate concentrations of 0.5, 2.91, and 3.5 μg/mL (2.9, 17.2, and 20.7 μM) were also able to induce a significant increase of MN frequency in human HepG2 cells after 4-h treatment in a study by Kasuba et al. (38); however, because HepG2 cells are cancerous human hepatocytes and are therefore characterized by an inherent genomic instability, MN results from these cells may not provide an adequate basis for comparison.

Unlike with glyphosate, a clear dose-dependent increase in MN frequency could be observed for all the three GBHs after both treatment regimens. Four-hour exposure to GBHs caused a statistically significant elevation of the MN frequency from 10 μM (Glyfos) and at 100 μM (Roundup Mega and Fozat 480), suggesting that co-formulants play a role not only in enhancing cell death but also in inducing genotoxic damage at non-cytotoxic concentrations. The more potent MN-inducing ability of Glyfos may be attributed to the POEA-content of this formulation, the direct DNA-damaging effect of which has already been established by previous toxicological studies (51, 52, 55, 56). Guilherme et al., exposing fish blood cells with Roundup formulation and its constituents, found that the genotoxic effect separately induced by POEA and glyphosate was not strengthened when the two substances were used in combination, ruling out a synergistic interaction between the ingredients of Roundup (52). Hao et al. demonstrated that POEA and Roundup exposure induce oxidative DNA lesions and other biochemical changes in human A549 cells, which were not detected in cells treated with glyphosate alone (14).

After 20-h exposure, although the difference in MN-inducing potential between the GBHs and glyphosate leveled out at 100 μM, Fozat 480 showed an increased genotoxic activity compared to other herbicides, as it already induced a statistically significant effect at 10 μM. This observation justifies our previous conclusion about the toxicity of non-declared adjuvant content of Fozat 480, which may be similar to that of Glyfos banned in Europe. Besides, exposure time seems to be an important factor in the clastogenic damage induced by glyphosate, as it could only have a significant effect after 20-h. This may be because glyphosate alone requires longer time to penetrate into cells and induce genomic damage, compared to being in a formulation where surfactant adjuvants would facilitate its entry by disrupting the cell membrane. This idea is partly corroborated by the study of Richard et al. that found glyphosate cytotoxicity increased with time (57), but contradicts with the report by Kasuba et al. who found significantly higher number of MN after 4-h than after 24-h in HepG2 cells exposed to glyphosate. However, they used metabolically active hepatocarcinoma-derived cell line that could result in the increased detoxification of glyphosate by 24-h (38). If the latter assumption is correct, we should have detected difference between the toxicity endpoints obtained with and without S9 treatment, especially after 20-h exposure; however, neither clear detoxification nor metabolic activation of any herbicides was observed in our study. Considering the lack of S9-dependent effects in the present study, we can conclude that metabolites have no increased clastogenic potential compared to the parent molecules. Finally, no significant changes in the proliferation index induced by both 4-h and 20-h exposure to all the tested herbicides could be detected, suggesting that neither toxicants interfere with mechanisms of cell division over the tested concentration range that confirms previous observations by Santovito et al. (37).

As with all studies, the current research also has certain limitations. First, the MN-inducing ability of co-formulants alone could not be measured due to limited information on the exact identity and concentration of the adjuvants, as well as other useful toxicity endpoints such as cell membrane permeabilization, mitochondrial potential, free radical levels, etc., were not examined, but may be the subject of future investigations. Second, our data exhibit some inter-experimental variability that may be due to the use of primary cell cultures obtained from blood samples of various donors. The different genetic background as well as lifestyle and environmental factors can strongly modify the susceptibility of individuals to genotoxic exposures and that variability is reflected in the variability of data between experiments. Increasing biological replicates could have reduced inter-experimental variability, and the validity of the results could have been improved by using machine-based automated MN scoring system. Third, CBMN assay is a well-established genotoxicity test but it has relatively low sensitivity and specificity to predict carcinogenicity, which has already been well-recognized (58–60); therefore, more sensitive, robust *in vitro* approaches with improved prediction of human carcinogenic risk may be needed. In addition, investigations with prolonged exposure times (>20-h) that better model realistic human exposure conditions may fill the knowledge gaps concerning chronic human health risks from accumulation of glyphosate in the food chain, because the presence of residual glyphosate in foodstuffs produced from glyphosate-treated crops, meat products from farmed animals that have consumed glyphosate-treated feed crops and contaminated drinking water constitute a continuous, albeit low-level, dietary exposure to the general population (61).

In conclusion, this is the first study that compares the MN-inducing potential of various glyphosate-based herbicides with their active ingredient in human peripheral white blood cells *in vitro*. Whilst glyphosate had a weak, but detectable genotoxic effect, GBHs exhibited both increased cytotoxic and genotoxic damage than the active substance that could be attributed to the effect of various adjuvants added to the formulations or to their interaction with glyphosate. Although some GBHs, that contain adjuvants with high toxicity, such as POEAs, have been banned in certain parts of the world (e.g., Glyfos), their counterparts with similar toxic properties are still on the market (e.g., Roundup Mega and Fozat 480). Thus, comprehensive toxicological assessment of co-formulants and complete formulations, together with the reconsideration of regulations allowing free access to GBHs are pressing challenges of the future. In addition, our findings underline the importance of biomonitoring studies based on micronucleus detection in order

to minimize the consequent cancer risk in populations exposed to glyphosate-based herbicides.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hungarian Ethical Committee for Medical Research. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KN and BÁ designed the experiments. KN, IS, RA, TS, and AA performed the majority of toxicity studies. RA, TS, and AA carried out the microscopic analyses. KN, RA, TS, and AA evaluated the data and interpreted the results. KN, RA, and BÁ wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.639143/full#supplementary-material

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Association Between Rare Earth Element Cerium and the Risk of Oral Cancer: A Case-Control Study in Southeast China

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Cerium (Ce), the most abundant of rare earth elements in the earth's crust, has received much health concerns due to its wide application in industry, agriculture, and medicine. The current study aims to evaluate whether there is an association between Ce exposures and the risk of developing oral cancer. Serum Ce level of 324 oral cancer patients and 650 matched healthy controls were measured by inductively coupled plasma mass spectrometry. Association between Ce level and the risk of oral cancer was estimated with an unconditional logistic regression model. Serum Ce concentrations in the oral cancer patients and controls were 0.57 (0.21–3.02) μg/L and 2.27 (0.72–4.26) μg/L, respectively. High level of Ce was associated with a decreased risk of oral cancer (OR: 0.60, 95% CI: 0.43–0.84). Stronger inverse associations between high level of Ce and oral cancer risk were observed among those with smoking (OR: 0.46, 95% CI: 0.27–0.79), drinking (OR: 0.50, 95% CI: 0.26–0.96), limited intake of leafy vegetables (OR: 0.40, 95% CI: 0.22–0.71) and fish (OR: 0.52, 95% CI: 0.33–0.83). There were significant multiplicative interactions between Ce level and alcohol drinking or intake of leafy vegetables and fish (all $P_{interaction}$ <0.05). This preliminary case-control study suggests an inverse association between high serum Ce level and the risk of oral cancer. Further prospective studies with a larger sample size are needed to confirm the findings.

Keywords: rare earth elements, cerium, oral cancer, inductively coupled plasma mass spectrometry, risk assessment

INTRODUCTION

Oral cancer is a common malignant tumor on the oral and maxillofacial. An estimated 354,864 new cases and 177,384 deaths from lip and oral cancers occurred globally in 2018, accounting for 2.0 and 1.9% of all new cancer cases and deaths (1). To date, there have been many studies on the etiology of oral cancer, among which tobacco, alcohol, and limited intake of fish and vegetables are the main known risk factors for oral cancer (2, 3). However, oral cancer still occurs in those with non-smoking, non-drinking and dietary balance, suggesting that there are other unknown factors associated with oral cancer.

Cerium (Ce) is the rare earth elements with the highest abundance in earth's crust (4). It was widely used in the field of high technologies and traditional industries such as in agriculture and the production of phosphors, alloys and catalysis with its unique trivalent state (5). Ingestion of contaminated food and water, direct uptake via medical administration, and occupational exposure are the potential sources for the transfer of cerium from the environment to the human body posing health risks (6–8).

Numerous studies indicated that Ce played a vital role in biological processes relevant to various cancers. Ce has been reported to exhibit antioxidant activity by scavenging ROS in healthy cells while acting as a pro-oxidant and having anti-cancer activity in cancer cells by inducing ROS formation (9). *In vitro* experiments demonstrated that CeO_2 nanoparticles induced significant oxidative stress leading to decreased viability of human lung cancer cells and colon cancer cells (10, 11). An *in vivo* study also revealed a new role of CeO_2 nanoparticles as a novel anti-angiogenic agent that attenuated tumor growth in a preclinical mouse model of ovarian cancer (12). Madero-Visbal et al. also reported that CeO_2 nanoparticles may decrease radiation-induced xerostomia and G-III dermatitis in head and neck cancer (13). However, most previous studies focused on cell or animal experiments. So far, limited data related to Ce and cancers are available in population-based on an epidemiological study, let alone the associations of Ce on oral cancer risk in southeastern China where a high yield of rare earth is produced. Therefore, the purpose of this study was (1) to measure the concentrations of Ce in the serum of oral cancer patients in comparison with the cancer-free controls; (2) to evaluate the association of serum levels of Ce with oral cancer risk.

TABLE 1 | Distribution of selected characteristics among patients with oral cancer and controls.

Variables	Cases (%) (n = 324)	Controls (%) (n = 650)	χ^2	P-value
Age(years)			0.41	0.524
<60	138 (42.59)	263 (40.46)		
≥60	186 (57.41)	387 (59.54)		
Gender			2.14	0.143
Male	202 (62.35)	436 (67.08)		
Female	122 (37.65)	214 (32.92)		
Race[a]				0.340
Han	321 (99.07)	648 (99.69)		
Others	3 (0.93)	2 (0.31)		
Education level			15.70	<0.001
Illiterate	32 (9.88)	90 (13.85)		
Primary and middle school	193 (59.56)	433 (66.61)		
High school and above	99 (30.56)	127 (19.54)		
Marital status			1.83	0.176
Married	300 (92.59)	616 (94.77)		
Others	24 (7.41)	34 (5.23)		
Residence			121.95	<0.001
Rural	164 (50.62)	546 (84.00)		
Urban	160 (49.38)	104 (16.00)		
Family history of cancer			9.00	0.003
No	275 (84.88)	593 (91.23)		
Yes	49 (15.12)	57 (8.77)		
Tobacco smoking			3.23	0.072
No	182 (56.17)	404 (62.15)		
Yes	142 (43.83)	246 (37.85)		
Alcohol drinking			15.19	<0.001
No	206 (63.58)	491 (75.54)		
Yes	118 (36.42)	159 (24.46)		
Leafy vegetables (per day)			28.00	<0.001
<2 times	139 (42.90)	170 (26.15)		
≥2 times	185 (57.10)	480 (73.85)		
Red meat (per week)			7.09	0.008
<3 times	96 (29.63)	142 (21.85)		
≥3 times	228 (70.37)	508 (78.15)		
Fish (per week)			27.38	<0.001
<3 times	185 (57.10)	256 (39.38)		
≥3 times	139 (42.90)	394 (60.62)		
Seafood (per week)			17.07	<0.001
<1 time	182 (56.17)	274 (42.15)		
≥1 time	142 (43.83)	376 (57.85)		
Eggs (per week)			2.97	0.085
<3 times	167 (51.54)	297 (45.69)		
≥3 times	157 (48.46)	353 (54.31)		

[a]*Fisher's exact test.*

METHODS

Study Participants

This case-control study was conducted in Fujian province, China, from September 2011 to January 2018. A total of 461 oral cancer patients were recruited from the First Affiliated Hospital of Fujian Medical University. Cases met the following inclusion criteria:

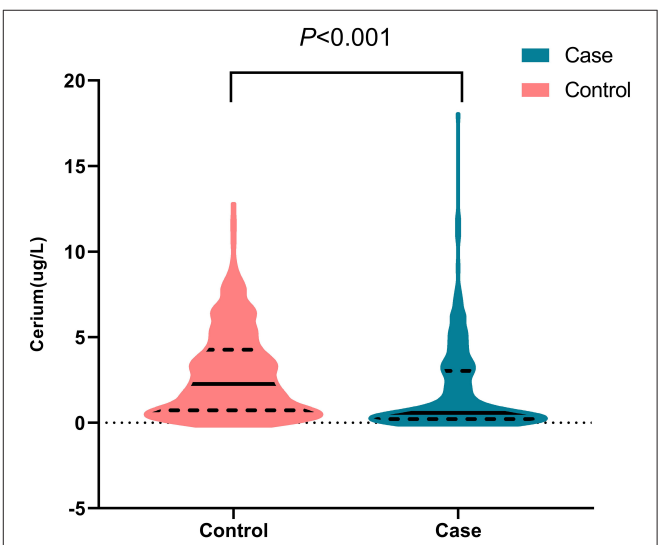

FIGURE 1 | Distribution of serum Ce in case and control groups.

(1) all cases were primary oral cancer patients, diagnosed after histological confirmation; (2) all cases reside in Fujian Province; (3) all cases aged 20 to 80 years. The exclusion criteria of cases included: (1) recurrent or metastasized oral cancer; (2) those who undertook radiotherapy or chemotherapy; (3) any history of severe systemic diseases such as severe liver and kidney dysfunction and AIDS; (4) long-term use of any dietary supplements. This left 324 (72.3%) patients in the final cohort for analysis.

During the study period, a control group of 650 individuals was randomly selected from the physical examination center of the same hospital who had no history of cancers. The healthy status was identified based on the results of physical examination. Cases were frequency matched to controls, in a ratio of about 1:2, considering both age and sex. The exclusion criteria were as follows: (1) those who were not in the same period as the case group; (2) those who were not from single households; (3) those who did not reside in Fujian Province, or those aged <20 or >80 years; (4) those who used long-term dietary supplements.

This study was performed according to the ethical principles of the Declaration of Helsinki and approved by the Institutional Review Board of Fujian Medical University (Approval number: 2011053).

TABLE 2 | ORs for the serum levels of Ce in relation to oral cancer.

Variables	Controls (%)	Cases (%)	*OR* (95%*CI*)	*OR* (95%*CI*)[a]
Ce (Continuous)	650 (100.00)	324 (100.00)	0.83 (0.77–0.88)	0.90 (0.84–0.96)
Ce(μg/L)				
<2.27(Low)	325 (50.00)	225 (69.44)	1.00	1.00
≥2.27(High)	325 (50.00)	99 (30.56)	0.44 (0.33–0.58)	0.60 (0.43–0.84)

[a]*Adjustment for age, gender, race, education level, marital status, family history of cancer, residence, tobacco smoking, alcohol drinking, and diet containing leafy vegetables, red meat, fish, eggs and seafood.*

Data Collection

Signed informed consents were provided from all participants. Data were collected through in-person interview using a standardized questionnaire which included the information about demographic characteristics, family history of cancer, smoking habits, alcohol consumption, as well as dietary intake. As for dietary data, the questionnaire contained five broad categories (red meat: pork/beef/lamb; fish; seafood: shrimp/crab/shellfish; eggs: chicken and duck eggs; leafy vegetables; dietary supplements). Then, participants were asked how often intake of each food item according to the following options: 3 times per day; 2 times per day; 1 time per day; 5–6 times per week, 3–4 times per week; 1–2 times per week; <1 time per week or not at all. And these questions about dietary habits were directed 1 year prior to cancer diagnosis or interview (for controls). The overall response rate of the questionnaire was 90.2% (96.1% for cases and 87.5% for controls). All participants were engaged in the study after being interviewed directly.

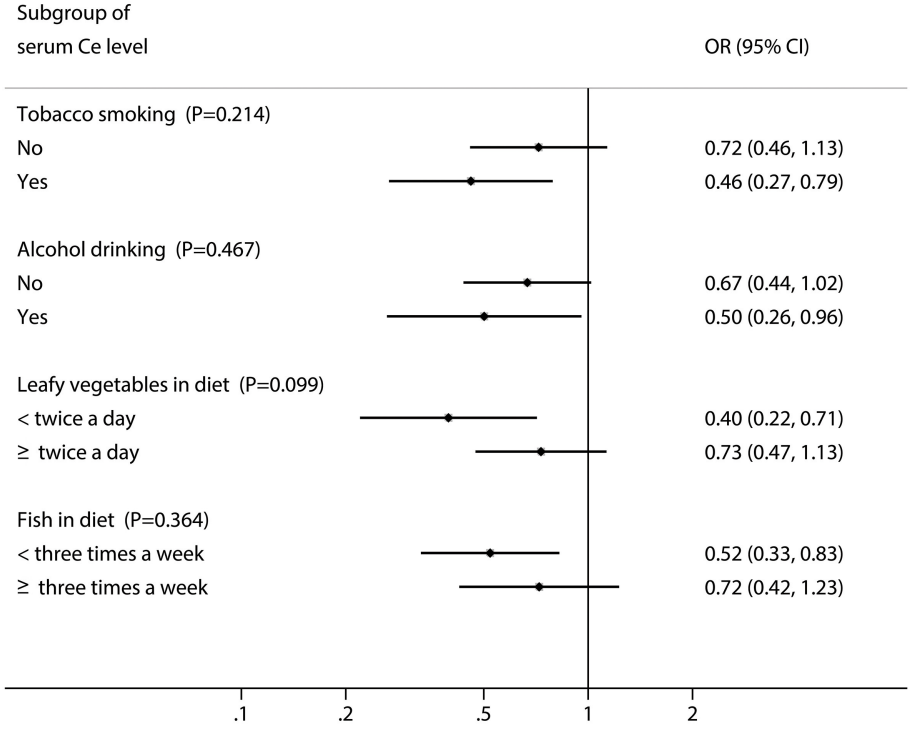

FIGURE 2 | Odds ratios (ORs) and 95% CIs for the serum Ce stratified by environmental factors.

Collection and Analysis of Blood Samples

Fasting venous blood samples (about 3–5 mL) for determining Ce levels of cases were collected on the second day of hospitalization prior to any drug treatment or examination. Then the control serum samples (about 3–5 mL) were from individuals seeking a routine health check up in the physical examination center. Then, all samples were centrifuged at 1,509 g for 10 min at 4°C, and serum was collected. Serum samples were immediately transferred and stored at −80 °C until needed for analysis.

Detailed laboratory methods for measurements had been described previously (14). Briefly, serum samples were digested by a microwave digestion system (PreeKem, China). Then, inductively coupled plasma massed spectrometry (ICP-MS, NexION 350X; Perkin-Elmer, USA) was used to determine serum Ce concentration. For the analytic quality control, human hair powders (GBW07601a, China) as internal standard reference materials were used to monitor the efficiency and accuracy of ICP-MS analysis. Each batch of samples contained at least two blanks and two standard reference materials. Meanwhile, 12.5% of the samples in each batch were randomly selected for parallel sample testing, and the relative standard deviation of parallel samples was <10%. Limits of detection were 0.0003 μg/L for Ce, and recovery of standard rare earth elements (accuracy) ranged from 89.1 to 92%.

Statistical Analysis

Chi-square tests or Fisher's exact tests were used to compare the distribution of demographic characteristics and environmental factors. The difference of serum Ce between cases and controls was assessed by the Wilcoxon rank-sum test and shown by violin charts. Associations between serum Ce and oral cancer were assessed by calculating odds ratios (ORs) and 95% confidence intervals (95%CIs) using unconditional logistic regression models. Moreover, stratified analyses and interaction terms were used to test the potential modification effects of environmental factors on the level of serum Ce for oral cancer. Additionally, the method proposed by Katsouyanni et al. (15) was utilized to develop a prediction model for oral cancer risk. The predictive ability of the models was evaluated using area under the receiver operating characteristic (ROC) curve (AUC). All tests were based on a 2-sided $P < 0.05$ as evidence of statistical significance. Data analysis was performed using R software version 3.6.0 or GraphPad prism8.0.

RESULTS

Table 1 shows the main characteristics of the 324 cases and 650 controls. The distributions of age, gender, race, marital status, tobacco smoking and intake of eggs were similar between the case

TABLE 3 | Combined effect of the serum levels of Ce and tobacco smoking, alcohol drinking or intake of leafy vegetables or fish for oral cancer.

Variables		Case (%)	Control (%)	Adjusted *OR* (95% *CI*)[a]	$P_{interaction}$
Ce (μg/L)	Tobacco smoking				
<2.27(Low)	Yes	105 (28.85)	117 (13.93)	1.00	
<2.27(Low)	No	120 (32.97)	208 (24.76)	0.49 (0.30–0.79)	0.003
≥2.27(High)	Yes	37 (10.16)	129 (15.36)	0.42 (0.25–0.70)	0.001
≥2.27(High)	No	62 (17.03)	196 (23.33)	0.38 (0.22–0.65)	<0.001
Ce × Tobacco smoking	–	–	0.70 (0.47–1.04)	0.081	
Ce (μg/L)	Alcohol drinking				
<2.27(Low)	Yes	89 (24.45)	90 (10.71)	1.00	
<2.27(Low)	No	136 (37.36)	235 (27.98)	0.44 (0.28–0.70)	0.001
≥2.27(High)	Yes	29 (7.97)	69 (8.21)	0.47 (0.26–0.86)	0.014
≥2.27(High)	No	70 (19.23)	256 (30.48)	0.30 (0.18–0.49)	<0.001
Ce × Alcohol drinking	–	–	0.56 (0.39–0.81)	0.002	
Ce (μg/L)	Leafy vegetables (per day)				
<2.27(Low)	<2 times	103 (28.30)	90 (10.71)	1.00	
<2.27(Low)	≥2 times	122 (33.52)	235 (27.98)	0.46 (0.31–0.69)	<0.001
≥2.27(High)	<2 times	36 (9.89)	80 (9.52)	0.52 (0.30–0.91)	0.022
≥2.27(High)	≥2 times	63 (17.31)	245 (29.17)	0.30 (0.19–0.48)	<0.001
Ce × Leafy vegetables	–	–	0.51 (0.35–0.74)	<0.001	
Ce (μg/L)	Fish (per week)				
<2.27(Low)	<3 times	127 (34.89)	114 (13.57)	1.00	
<2.27(Low)	≥3 times	98 (26.92)	211 (25.12)	0.37 (0.24–0.57)	<0.001
≥2.27(High)	<3 times	58 (15.93)	142 (16.90)	0.46 (0.29–0.71)	0.001
≥2.27(High)	≥3 times	41 (11.26)	183 (21.79)	0.32 (0.20–0.51)	<0.001
Ce × Fish	–	–	0.57 (0.37–0.86)	0.008	

[a]*Adjustment for age, gender, race, education level, marital status, family history of cancer, residence, tobacco smoking, alcohol drinking, and diet containing leafy vegetables, red meat, fish, eggs and seafood.*

TABLE 4 | Multivariate unconditional logistic regression analysis of influencing factors of oral cancer.

Variables	Model-1[a]		Model-2[b]	
	P	*OR* (95%*CI*)	*P*	*OR* (95%*CI*)
Tobacco smoking				
No		1.00		1.00
Yes	0.018	1.64 (1.09–2.46)	0.019	1.64 (1.09–2.48)
Alcohol drinking				
No		1.00		1.00
Yes	<0.001	2.10 (1.42–3.11)	<0.001	2.03 (1.37–3.01)
Leafy vegetables (per day)				
<2 times		1.00		1.00
≥2 times	<0.001	0.49 (0.36–0.68)	<0.001	0.50 (0.36–0.69)
Red meat (per week)				
<3 times		1.00		1.00
≥3 times	0.097	0.73 (0.51–1.06)	0.072	0.71 (0.49–1.03)
Fish (per week)				
<3 times		1.00		1.00
≥3 times	<0.001	0.48 (0.34–0.67)	<0.001	0.48 (0.35–0.67)
Seafood (per week)				
<1 time		1.00		1.00
≥1 time	0.002	0.59 (0.42–0.83)	0.002	0.58 (0.41–0.82)
Eggs (per week)				
<3 times		1.00		1.00
≥3 times	0.257	0.83 (0.61–1.14)	0.220	0.82 (0.60–1.13)
Ce(μg/L)				
<2.27 (Low)		–		1.00
≥2.27 (High)	–	–	0.003	0.60 (0.42–0.84)
AIC		1040.50		1033.82

[a]*Adjustment for age, gender, race, education level, marital status, family history of cancer, residence.*
[b]*Additional adjustment of serum Ce.*

and control groups ($P > 0.05$). However, significant differences were observed with regard to education levels, residence, family history of cancer, alcohol drinking and intake of fish, seafood, leafy vegetables and red meat (all $P < 0.05$).

As is shown in **Figure 1**, the median (quartile25-quartile75) of the concentrations of serum Ce in the oral cancer patients and controls were 0.57(0.21–3.02) μg/L and 2.27(0.72–4.26) μg/L, respectively. Moreover, serum Ce was significantly different between oral cancer patients and controls ($P < 0.001$). When serum Ce was regarded as a continuous variable, an inverse association was observed between serum Ce and the risk of oral cancer [the *OR* was 0.90 (95% *CI*: 0.84, 0.96) for a one-unit increase]. Then, when serum Ce levels were dichotomized into high and low levels, using the median of the serum Ce concentrations in the control group as the cutoffs, high levels of serum Ce was associated with a decreased risk of oral cancer after other potential confounders were controlled (*OR*: 0.60, 95% *CI*: 0.43–0.84, **Table 2**).

When further stratified by environmental factors, as displayed by **Figure 2**, stronger inverse associations were observed between the high levels of serum Ce and oral cancer risk among those with smoking (*OR*: 0.46, 95% *CI*: 0.27–0.79), drinking (*OR*: 0.50, 95% *CI*: 0.26–0.96), limited intake of leafy vegetables (*OR*: 0.40, 95% *CI*: 0.22–0.71) and fish (*OR*: 0.52, 95% *CI*: 0.33–0.83). Additionally, there were significant multiplicative interactions between the serum levels of Ce and alcohol drinking or intake of leafy vegetables and fish for oral cancer (all $P_{interaction} < 0.05$, **Table 3**).

Subsequently, a multivariate logistic regression analysis was performed to develop different models by incorporating serum Ce and environmental factors (**Table 4**). Compared with model-1, model-2 had the higher discriminatory ability for oral cancer risk, with the lower Akaike information criterion (AIC) value (1033.82). Moreover, the area under the curve of model-2 was larger than that of model-1 ($P = 0.015$, **Figure 3**).

DISCUSSION

In this relatively large-scale case-control study, we systematically evaluated the serum Ce and risk of oral cancer using an advanced analytical technique (ICP-MS). The results revealed that high serum Ce was significantly associated with decreased risk of oral cancer. Interestingly, the inverse association was more obvious among tobacco smokers, alcohol drinkers or those consumed fewer leafy vegetables or fish. Additionally, significant multiplicative interactions between serum levels of Ce and alcohol drinking or intake of leafy vegetables or fish for oral cancer were also observed.

Several studies have revealed the anticancer activity of Ce in many types of cancer cells. Giri et al. (12) showed that increase of nanoceria expression was associated with the increased ability to inhibit metastasis and angiogenesis in ovarian cancer cells, thus contributing to attenuate ovarian tumor growth (12). Moreover, Ce was also found to exhibit toxicity in human colon cancer cells, neuroblastoma cells, and fibrosarcoma cells through generating ROS which induces apoptotic cell death (11, 16, 17). We speculated that the association of high serum Ce levels with decreased risk of oral cancer may be attributed to the anticancer potential of Ce in cancer cells. Because of the increased rate of glycolysis and lactic acid production, cancer cells develop relatively a higher acidic environment than healthy cells. The lower pH environment causes loss of antioxidant (cytoprotective) ability of Ce, instead it behaves as a pro-oxidant, which may facilitate the production of ROS to cause tumor cell apoptosis (18, 19). Interestingly, when stratified by smoking status, the protective effect of high levels of serum Ce in smokers on oral cancer was higher than that in non-smokers. It was well-established that a large number of free radicals in cigarette smoke attacked the genetic material of cells directly or indirectly, and played a crucial role in the process of inducing and promoting cancer (20). Whereas, Ce could prevent the formation of free radicals and ROS by mimicking the functions of antioxidant enzymes such as superoxide dismutase and catalase (21). A possible mechanism for this due to the fact that high levels of serum Ce can interfere with the production of smoking-induced free radicals through

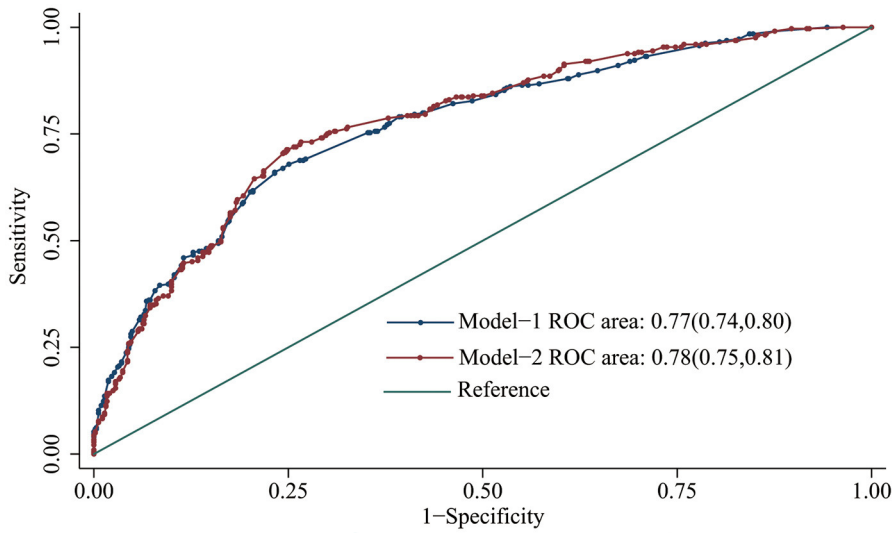

FIGURE 3 | Receiver operating characteristic curve of different multifactor models for predicting oral cancer risk.

antioxidation. Further research is still needed to explore the exact mechanism.

Moreover, our data revealed that the association between serum Ce levels and the risk of oral cancer was modified by alcohol drinking, with multiplicative interactions between them. Alcohol intake was found to reduce the immune function of the body, and caused the increased permeability of oral mucosa to carcinogens (22, 23). Data from a chronic toxicity test indicated that the oral administration of Ce at low and moderate doses could significantly improve the immunity of male rats by increasing white blood cells (WBCs) and the total lymphocyte counts (24). Therefore, a possible explanation is that Ce may reduce the risk of oral cancer caused by alcohol through strengthening the immune system of the human body.

Additionally, the result showed that there was a multiplicative interaction between serum cerium and green leafy vegetable intake, and the protective effect was the strongest in the group of high serum cerium level and high vegetable intake. On the one hand, some studies have confirmed that a diet adequate in vegetables may protect against oral cancer (25, 26). Although the mechanisms by which vegetables may confer protection against cancer is not entirely clear, cruciferous vegetables are important sources of glucosinolates, whose major breakdown products (isothiocyanates and indoles) have been demonstrated to have anti-carcinogenic properties *in vitro* as well as in animal studies (27, 28). On the other hand, cerium has the effect of antioxidation. Therefore, serum cerium and adequate vegetable intake play a synergistic role in the pathogenesis of oral cancer.

There are also limitations of the current study that merit mention. First, we only collect blood samples at the first visit, and the exposure time reflected might not be enough to cover the latency of oral cancer development (29), which precluded us from firmly establishing a temporal association between serum Ce and oral cancer. Therefore, although we hypothesized that the concentration of Ce measured in our study reflected the level of pre-cancerous cancer, we cannot rule out the possibility that the concentration is affected by oral cancer. Consequently, further prospective studies are needed to confirm our findings. Second, Ce can be present in some solid or solution in the form of a positive divalent, tetravalent, or mixed valence (30). Nevertheless, the detection methods used in this study cannot determine the valence state of Ce, hopefully, which could be distinguished in future studies.

CONCLUSION

In conclusion, this preliminary case-control study reveals lower serum Ce levels in patients with oral cancer than that of healthy controls, and suggests an inverse association between high serum Ce levels on the risk of oral cancer. This association may be modified by smoking, alcohol drinking, and dietary patterns. Much more extensive research is warranted to confirm these possible associations and to clarify their mechanisms, which will promote the application of Ce in the diagnosis and treatment of oral cancer and provide a new strategy for improving tumor prevention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Fujian Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC and BH participated in the design of the study. LL, BS, YQ, ZZ, and YH were responsible for recruitment and interview participants. LP and XZ contributed to samples collection. JW, JC, and JL performed laboratory experiments. JL, FL, and LY analyzed the data. BH, JW, and FC wrote the manuscript, which was revised by all authors. All authors contributed to the article and approved the submitted version.

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Environmental Tobacco Smoke in Occupational Settings: Effect and Susceptibility Biomarkers in Workers From Lisbon Restaurants and Bars

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Environmental tobacco smoke (ETS) has been recognized as a major health hazard by environmental and public health authorities worldwide. In Portugal, smoke-free laws are in force for some years, banning smoking in most indoor public spaces. However, in hospitality venues such as restaurants and bars, owners can still choose between a total smoke-free policy or a partial smoking restriction with designated smoking areas, if adequate reinforced ventilation systems are implemented. Despite that, a previous study showed that workers remained continuously exposed to higher ETS pollution in Lisbon restaurants and bars where smoking was still allowed, comparatively to total smoke-free venues. This was assessed by measurements of indoor $PM_{2.5}$ and urinary cotinine, a biomarkers of tobacco smoke exposure, demonstrating that partial smoking restrictions do not effectively protect workers from ETS. The aim of the present work was to characterize effect and susceptibility biomarkers in non-smokers from those hospitality venues occupationally exposed to ETS comparatively to non-exposed ones. A group of smokers was also included for comparison. The sister chromatid exchange (SCE), micronucleus (MN) and comet assays in whole peripheral blood lymphocytes (PBLs) and the micronucleus assay in exfoliated buccal cells, were used as biomarkers of genotoxicity. Furthermore, a comet assay after *ex vivo* challenge of leukocytes with an alkylating agent, ethyl methanesulfonate (EMS), was used to analyze the repair capacity of those cells. Genetic polymorphisms in genes associated with metabolism and DNA repair were also included. The results showed no clear association between occupational exposure to ETS and the induction of genotoxicity. Interestingly, the leukocytes from non-smoking ETS-exposed individuals displayed lower DNA damage levels in response to the *ex vivo* EMS challenge, in comparison to those from non-exposed workers, suggesting a possible adaptive response. The contribution of individual susceptibility to the effect biomarkers studied was unclear, deserving further investigation.

Keywords: second-hand smoke, occupational exposure, human biomonitoring, genotoxicity, challenge assay

INTRODUCTION

Environmental tobacco smoke (ETS), also known as second-hand smoke (SHS), passive smoke or involuntary smoke is a widespread indoor pollutant of significant public health concern, and a major risk factor for lung cancer and other diseases (1–5). The mutagenic and carcinogenic effects of tobacco smoke have been clearly demonstrated (1, 2, 6, 7) and its adverse effects are not limited to smokers, but affect also environmentally and occupationally exposed non-smokers, since it is present in all places where smoking takes place (2). ETS is a significant source of a complex mixture of hundreds of hazardous substances comprising the smoke emitted from the burning tip of a cigarette (or other burned tobacco product) between puffs (sidestream smoke, SM), the smoke exhaled by the smoker (mainstream smoke, MS), and also the compounds diffused through the cigarette paper wrapper (1, 2, 8). ETS is classified as carcinogenic to humans (group 1) by the International Agency for Research on Cancer (1, 2), based on a clear evidence of a causal association between exposure of non-smokers and cancer. Because of its rapid dilution and dispersion into the indoor environment, the concentrations of individual ETS constituents can vary with time and environmental conditions (1, 2), and currently there is no safe level of exposure to ETS (9). While social ETS exposure can be controllable (an individual may avoid to be in places where smokers are present), employees, however, have little or no influence over ETS and may be exposed for a large part of their working day (10). Occupational exposure to ETS was associated with an increase of 16–19% in the risk of lung cancer among never-smokers (1). Comprehensive smoke-free laws offer the only effective means of eliminating the risks associated with ETS (9). In fact, to protect people from ETS, since 2005, smoke-free policies have been expanding worldwide covering indoor public places and workplaces, including hospitality venues (9, 11, 12) and overall have been highly effective in reducing the exposure to constituents of ETS (5, 11, 13, 14), as well as decreasing ETS-related diseases (12, 15), particularly when complete smoke-free ban is applied. Nonetheless, ETS remains a common indoor air pollutant, especially in indoor leisure areas including restaurants, bars, nightclubs and casinos (9, 16–18). In Portugal, the law that prohibits smoking in most indoor public spaces and workplaces was introduced in 2008, but in some cases, such as restaurants and bars, partial smoking restrictions are applied, and smoking is still allowed in separate designated smoking areas if adequate reinforced ventilation systems are implemented. Whether the health of the ETS-exposed workers is affected remained an open question. A previous work by Pacheco et al. (17) showed that ETS indoor pollution, estimated by the concentration of particulate matter ($PM_{2.5}$), was consistently higher in restaurants where smoking was still allowed, comparatively to non-smoking restaurants and canteens (total smoke-free). In addition, the measurement of a biomarker of tobacco smoke exposure, i.e., cotinine, a metabolite of nicotine, confirmed the employees' exposure to ETS. Although all workers exhibited normal lung function, a proteomics approach identified differentially expressed proteins in the plasma of those ETS-exposed non-smoking workers, suggestive of alterations that may precede the first symptoms of tobacco-related diseases (19).

It is acknowledged that many substances contained in cigarette smoke are genotoxic and therefore genotoxicity biomarkers are good biomarkers to assess early effects from exposure to tobacco smoke (8, 20, 21), including ETS. The genotoxicity of ETS exposure has been addressed in few *in-vitro*, *in-vivo* and biomonitoring studies (8). In humans, environmental room exposure studies using fresh diluted sidestream smoke as a surrogate to estimate the effect of ETS exposure showed a slightly increased urinary mutagenicity (22) and DNA damage (23) in non-smoking voluntaries. Importantly, some studies reported the presence of DNA adducts (24), protein adducts (25–28) and urinary metabolites of carcinogens (29–31) after ETS exposure. On the other hand, biomonitoring studies evidencing the genotoxic effects of ETS on humans are scarce. While for chromosome instability results were predominantly negative (32–36), for the induction of DNA strand breaks, both positive (37, 38) and negative (39) results are described, although in the majority of those studies cotinine measurements confirmed the ETS exposure. A more evident genotoxic effect of ETS exposure appears to happen in children. A marginally significant increases of sister chromatid exchange (27), micronucleus (25, 40) and DNA damage measured with the comet assay (41–43) was reported. In the occupational settings, the impact of ETS on the genotoxicity biomarkers remains to be clarified.

The aim of the present work was to characterize the local and systemic genotoxic effects induced by occupational exposure to ETS in non-smoking workers from Lisbon restaurants and bars and to assess whether the genetic susceptibility could influence the observed effects. The sister chromatid exchange, micronucleus and comet assays in PBLs and the micronucleus assay in exfoliated buccal cells, were used to assess DNA and chromosome damage in ETS-exposed workers comparatively to non-exposed workers from the previously characterized hospitality venues (17, 19). A group of smokers working in the same venues was also included for comparison. In addition, the capacity of leukocytes to repair DNA lesions was estimated by the comet assay following their *ex vivo* exposure to an alkylating agent, ethyl methanesulfonate. Because several studies have evidenced the influence of genetic polymorphisms in genes encoding for metabolizing enzymes or DNA repair proteins on smoking-associated biomarkers, genotoxicity biomarkers and cancer predisposition (20, 44–50), some relevant susceptibility biomarkers were also studied. These included polymorphisms in metabolism (*GSTP1*^{105}, *GSTM1,* and *GSTT1*) and DNA repair (*hOGG1*^{326}, *XRCC1*^{194}, *XRCC1*^{399}, *XRCC3*^{241}, *NBN*^{185}, *PARP1*^{762}) genes.

MATERIALS AND METHODS

Workplace Characterization and Study Population

Among leisure establishments in Lisbon, restaurants and bars/discotheques were preselected based on a convenience sample. Accordingly, 58 main venues' owners were invited

to participate in the study by letter and personal approach. After detailed information of project objectives, 25 agreed to participate in the study. Venues were classified as smoke-free (SFre), smoking (Sre) and mixed restaurants and bars with both smoking (Sro) and non-smoking rooms (NSro), as previously described by Pacheco et al. (17). In addition, four public institutions canteens (Cant) where smoking was not allowed were also included. ETS was assessed by monitoring the level of indoor air contaminants, namely, particulate matter ($PM_{2.5}$), CO and CO_2 in all venues and a full description of the methods and results obtained can be found elsewhere (17, 19).

To estimate the adequate sample size of the study groups, a power analysis was performed based on the frequency of micronuclei (MN) in lymphocytes, a sensitive biomarker of an early biological effect. Based on published and our own data from control groups, the mean frequency of MN was expected to be 7.0/1,000 cells and the SD = 3.0. An 80% power is generally considered as acceptable (51). To obtain a two-tailed *p*-value of 0.05 and a difference between the exposed and control groups that corresponds to a 25% higher mean level of MN among the exposed, a minimum of 49 subjects would be needed in each group; if the difference increases to 30%, 33 subjects would be needed. Despite the estimates made and the invitation to participate to a larger number of employees, only 97 accepted to provide blood and buccal cells samples for genotoxic assessment.

Ethics approval for this study was secured from Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) ethics committee, Lisbon. Each potential participant was informed about the procedures and the objectives of the study and those who accepted to participate provided written informed consent for the collection and utilization of biological specimens. During the medical surveillance phase of the study, each subject was interviewed to evaluate clinical history, demographic and lifestyle information, particularly about smoking habits (including amount, frequency, and duration of smoking) or self-reported exposure to ETS at home. According to the inclusion criteria, healthy subjects with more than 18 years, who worked in the above referred hospitality venues for more than 9 h per week and for at least 1 month at the current workplace were included in the study. Excluded were the individuals submitted to X-rays, blood transfusion or surgery between 0 and 2 months before the study and those who suffer or had suffered from cancer. Thus, from the 97 volunteers preselected, 81 were included in effect and susceptibility biomarkers analysis. Detailed contextual data can be found in Pacheco et al. (19).

Workers were separated into three study groups according to smoking status and ETS occupational exposure as follow: a group of non-smoking workers (NSW, $n = 62$) that was subdivided according to ETS exposure on the workplace into the ETS-exposed group (E, $n = 29$) including workers from Sre or mixed restaurants and bars and the non-exposed group (NE, $n = 33$) including workers from SFre and Cant; a group of smoking workers (SW, $n = 19$) containing workers from SFre and from Sre or mixed venues (17).

Human exposure to ETS and confirmation of smoking habits had been previously assessed by urinary cotinine levels, allowing to discriminate between smokers and non-smokers and between non-smokers exposed and not exposed to ETS. A full description of the methods and results obtained can be found elsewhere (17, 19).

Biological Samples Collection

Following the interview and medical examination, biological samples were collected and coded to ensure their anonymization. For effect biomarkers characterization, peripheral blood and buccal epithelial cells samples were collected from each subject by medical personnel. Two mL of peripheral blood was collected by venipuncture into heparin-coated tubes and were processed within 2–3 h for SCE, MN and comet assays. Each subject was then asked to rinse the mouth twice with water and buccal epithelial cells were collected by gently scraping the oral mucosa of the inner lining of both cheeks with a plastic spatula. For genotyping of genetic polymorphisms, 2 mL of peripheral blood was collected into EDTA tubes, also by venipuncture.

The Alkaline Comet Assay in Peripheral Blood Lymphocytes

The alkaline version of the comet assay was used to evaluate DNA damage in PBLs from each subject and was carried out as described elsewhere (52), with some modifications. Briefly, 20 μL of whole blood was added to a 1 ml phosphate-buffered saline (PBS, Gibco-Invitrogen, Carlsbad, CA). Cells were pelleted and 40 μL were embedded in 0.7% low-melting point agarose (Sigma, St. Louis, MO) and then dropped onto microscope slides pre-coated with 1% agarose (Amersham Biosciences, Uppsala, Sweden) and covered with a coverslip for about 10 min, at 4°C. Simultaneously, to test the response of PBLs to an *ex vivo* challenge, 20 μL of whole blood was added to 1 ml PBS (Gibco-Invitrogen) and exposed to 32 mM of ethyl methanesulfonate (EMS, Sigma), incubated at 37°C for 30 min, and equally processed. After gel solidification, coverslips were removed and slides were immersed in freshly prepared ice-cold lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA.H_20$, 10 mM Tris HCL, NaOH, pH 10) with 10% Dimethyl Sulfoxide (DMSO, Sigma) and 1% Triton X-100 (Sigma), for 1 h, at 4°C, in the dark. After lysis, slides were placed on a horizontal electrophoresis tank in an ice bath, immersed in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA.2H2O, pH>13) in the dark, for 20 min, to allow DNA unwinding. Electrophoresis was then conducted at 25 V (∼0.74 V/cm, 300 mA), at 4°C for 20 min. Then, slides were rinsed with the neutralization buffer (0.4 M Tris–HCl, pH 7.5), stained with 125 μg/mL ethidium bromide (Sigma), covered with a coverslip, and kept in a dark, moist chamber. Two slides were prepared for each subject and a "blind" scorer examined 50 randomly selected cells from each slide (100 cells/subject) using a 200× magnification in a Axioplan2 imaging epifluorescence microscope (Carl Zeiss Microscopy, Göttingen, Germany) with an image analysis system (Comet Imager 2.2 Software, MetaSystems, Altlussheim, Germany). The mean percentage of DNA in the nucleoids tail (tail DNA,%) and the tail length (TL) were calculated for each worker.

Micronucleus Analyses in Peripheral Blood Lymphocytes

The Cytokinesis-blocked micronucleus assay (CBMN) was carried out as described elsewhere (53) with minor modifications. Briefly, whole blood samples (0.5 mL) from each subject were cultured in 4.5 mL RPMI-1640 medium with L- Glutamate (Gibco-Invitrogen) supplemented with Fetal Bovine Serum (25%, Sigma), phytohemaglutinin (2.5%, Gibco-Invitrogen), Penicillin-Streptomycin (1.5% Gibco-Invitrogen) and sodium heparin (0.5%, B. Braun Medical, Germany). Duplicate cultures from each subject were incubated at 37°C, for 68 h. Cytokinesis was blocked at 44 h of incubation by adding 5 μg/mL of cytochalasin B (Sigma-Aldrich, St. Louis, MO). After the 68 h of incubation, cells were harvested by treatment with a hypotonic solution (0.1 M KCl), at 37°C, followed by fixation (methanol: acetic acid, 3:1). Cells were immediately dropped onto microscope slides using cytocentrifugation, air-dried and stained with 4% Giemsa (Merck, Darmstadt, Germany) in pH 6.8 phosphate buffer. MN were blindly scored, under a bright field microscope (Axioskop 2 Plus, Zeiss, Germany) with a 400× magnification, and identified according to published criteria (54). From each subject at least one thousand binucleated cells (BC) with well-preserved cytoplasm (500 per replicate culture) were analyzed and the frequency of micronucleated binucleated cells per 1000 binucleated cells (MNBC/1000 BC) was calculated and represented as the mean number of MNBC/1000 BC ± SD. The proportion of mono- (MC), bi- (BC) or multinucleate cells (MTC) was determined in a total of 1,000 cells and the cytokinesis-block proliferation index (CBPI) was calculated as follows (55):

$$CBPI = (MC + 2BC + 3MTC)/\text{Total Cells}.$$

Sister-Chromatid Exchange Analyses in Peripheral Blood Lymphocytes

For the SCE analysis in PBLs, cultures were established in duplicate as described previously (56), with minor modifications. One mL of whole blood were added to 9 mL RPMI-1640 culture medium supplemented as described above for the CBMN. Bromodeoxyuridine (BrdU, Sigma) was added to a final concentration of 10 μg/mL and incubated at 37°C for 56 h, in the dark. Cultures were treated with 0.1 mg/mL colcemid (Gibco-Invitrogen), 1 h prior to harvesting. Cells were processed through hypotonic treatment (0.075 M KCl) and fixation with methanol:acetic acid (3:1). Slides were prepared, air-dried and stained using the fluorescence plus Giemsa method (56, 57) for differential sister chromatids staining. For each subject, SCEs were analyzed in 50 second-division metaphases from two cultures, on coded slides, to determine the number of SCE per cell, and mean and standard deviation of the SCE counts per cell were calculated. The number of high frequency cells (HFCs) for each subject was determined as the proportion of metaphases whose SCE frequency exceeds the 95th percentile of the SCE distribution in the NE group, which was defined as those with a count of 14 or more SCEs.

Micronucleus Analysis in Buccal Exfoliated Epithelial Cells

Buccal exfoliated epithelial cells were smeared onto slides, air-dried and fixed in 80% cold methanol, for 20 min (58). Slides were stained according to Feulgen's technique (59) without cytoplasm counterstain. Two thousand cells were scored on two slides (one from each cheek) from each individual (1000 cells per slide) based on published criteria (58, 59). Only cells containing an intact nucleus that was neither clumped nor overlapping were included in the analysis. The frequencies of micronucleated cells (MNC) and nuclear buds (NBUD) per 1000 cells were determined for each subject and represented as the mean number of MNC/1000 cells ± SD and NBUD/1000 cells ± SD, respectively.

Genetic Polymorphisms in Metabolism and DNA Repair Genes

Genomic DNA was isolated from whole blood samples of the workers with the MagNA Pure LC DNA Isolation Kit (Roche Applied Science, Germany) following the manufacturer's instructions. DNA samples were stored at −20°C until analysis. Genetic polymorphisms in metabolism (*GSTP1*^{105}, *GSTM1,* and *GSTT1*) and DNA repair (*hOGG1*^{326}, *XRCC1*^{194}, *XRCC1*^{399}, *XRCC3*^{241}, *NBN*^{185}, and *PARP1*^{762}) genes were analyzed using PCR-based assays, according to published methods with minor modifications. *GSTT1* and *GSTM1* genotype analysis was performed in the same reaction, in a multiplex PCR (60). DNA amplification by PCR with specific primers flanking the polymorphism, followed by enzymatic restriction and fragments' analysis by gel electrophoresis, i.e., the PCR-RFLP method, was used to characterize the following polymorphisms: *GSTP1*^{105} (61), *hOGG1*^{326} (62), *XRCC1*^{194}(63), *XRCC1*^{399}(63), *XRCC3*^{241}(64), *NBN*^{185}(65), and *PARP1*^{762} (66). The details of the primers, restriction enzymes, and PCR conditions are described in the **Supplementary Table 1**.

Statistical Analysis

All statistical analyses were conduct using the IBM SPSS 17.0 for Windows statistical package. The level of significance considered was $p < 0.05$. The distribution of variables in total population and divided by groups was compared with the normal distribution by means of the Kolmogorov–Smirnov test. The studied variables MNBC/1000 BC cells, CBPI, MNC/1000 cells, NBUD/1000 Cells, SCE frequencies,% tail DNA, and TL departed significantly from normality and therefore non-parametric tests were applied. Chi-square test was applied to compare the frequency of MNBC/1000 BC and the frequency of MNC/1000 cells or NBUD/1000 cells in buccal epithelial cells between exposure groups. The frequencies of SCEs, HFCs, CPBI,% tail DNA and TL from each group were compared using the non-parametric Mann-Whitney U-test. The relationship between the biomarkers of early biological effects (MN and SCE frequencies, % tail DNA) and the duration of exposure to cigarette smoke (active and passive), cigarette consumption (number of cigarettes per day), cotinine concentration and age was explored by Spearman's correlation analysis. The same analysis was also used to explore correlations between the several effect biomarkers analyzed. The

effect of gender on the cytogenetic parameters or in the genetic polymorphism was assessed by the Mann–Whitney *U*-test. Regarding the genetic polymorphisms, deviation from Hardy–Weinberg (HW) equilibrium was assessed with the Chi-Square-test. Statistical analysis using Pearson Chi-Square (2-sided), or two-sided Fisher's exact test were applied to assess differences between studied groups concerning allele distributions. To assess the influence of the genotype on each effect biomarker, non-parametric tests (Kruskal–Wallis or Mann–Whitney *U*-test) were applied. Two types of comparisons were made: the influence of the gene variants on the biomarker level within each exposure group and inter-group comparison according to each allelic variant. Due to the low number of homozygous variant carriers of $XRCC1^{194}$, $XRCC1^{399}$, $PARP1^{762}$, and $XRCC3^{241}$, all subjects harboring variant alleles (homozygous and heterozygous) were pooled together.

RESULTS

Characteristics of the Study Population

General characteristics of the studied groups are described in **Table 1**. The studied population consisted of 81 workers, the majority being males (74%). The mean age was 45.2 ± 12.2 years for the NE group, 37.2 ± 10.8 years for the E group and 39.1 ± 11.1 years for SW. Mean age in the NE group was significantly higher than in E group ($p = 0.008$, unpaired *t*-test). Most employees stated to work at least 40 h per week (NE: 48.9 h ± 12.3; E: 39.1 ± 9.7 h; SW: 47.5 ± 12.6 h). Mean h per week in the E group was significantly lower than in NE group ($p < 0.0001$, Mann–Whitney *U*-test) and in SW group ($p = 0.0024$, Mann–Whitney *U*-test). The average months in the current job was higher in NE group (155.6 ± 151.4) in comparison with the E group (74.9 ± 91.2) and SW group (67.6 ± 54.3). Regarding smokers (SW), the average number of cigarettes smoked per day was 16.5 ± 7.5 (range 3–30), and the mean number of years as a smoker was 22.9 ± 10.9 (range 3–49). Most workers declared not being exposed to ETS out of the work, namely at home, and have no other professional activity where they could be occupationally exposed to ETS. Biological monitoring of ETS exposure was assessed by measurement of the urinary cotinine concentration, a metabolite of nicotine, using gas chromatography–mass spectrometry, as previously reported (17). As expected, the cotinine level was significantly increased in SW comparatively to NSW ($p < 0.0001$, Mann–Whitney *U*-test). The cotinine values obtained for non-smokers fall in the range considered involuntary exposure (17, 67). Among them, the mean cotinine level measured in the E group (7.98 ± 7.26 ng/mL) was significantly higher ($p = 0.0005$, Mann–Whitney *U*-test) than in the NE workers group (2.23 ± 4.31 ng/mL, the majority being below the level of quantification).

Biomarkers of Early Biological Effects

The results of the cytogenetic and DNA damage effect biomarkers studied are presented in **Tables 2**, **3**. In peripheral blood lymphocytes, no significant differences in the mean frequency of SCE/cell and in the level of HFCs were observed between the ETS-exposed (E) and NE groups. On the other hand, when considering the effect of smoking, a significantly higher percentage of HFCs was found in SW, comparatively to NE ($p = 0.003$, Mann–Whitney *U*-test) or to ETS-exposed groups ($p = 0.016$, Mann–Whitney *U*-test), although these differences were not detected when similar comparisons were made using the mean frequencies of SCEs (**Table 2**). The mean frequency of MNBC per 1000 BC was significantly different in the E as compared to the NE group ($p = 0.004$, Chi-square test) (**Table 2**). Unexpectedly, non-smoking workers exposed to ETS showed a 27.3% reduction in the frequency of MNBC in PBLs, as compared to non-smoking NE workers. Also, the SW group presented a significantly higher frequency of MNBC as compared with the ETS-exposed group ($p = 0.001$, Chi-square test), but no significant difference was detected between the group of SW and the NE group ($p = 0.53$, Fisher's Exact Test), both displaying a similar frequency of MNBC.

As to the level of DNA damage assessed by the comet assay, no differences in the percentage of DNA in tail or in the tail length were observed neither between the E and NE groups nor between SW and NSW groups (**Table 3**). In respect to the *ex vivo* challenge assay with EMS, the data show that both parameters, tail DNA and TL, were significantly different in ETS-exposed comparatively to the NE group ($p < 0.001$, Mann–Whitney *U*-test). The level of EMS-induced DNA damage was 26.6% lower in the E group, as compared to the NE group of workers. The individual values of tail DNA for each worker, from E or NE groups, is shown in **Figure 1**. After the *ex vivo* EMS challenge assay, the distribution of the data points from the E workers is always under a threshold of 36%, while NE individuals show a wider range of DNA damage induction, up to 57% (**Figure 1**). When comparing the SW with the ETS-exposed group, a significant difference was also observed ($p < 0.01$, Mann–Whitney *U*-test), with the E group presenting the lowest values of tail DNA and TL in challenged lymphocytes; no difference was observed between the SW and the NE groups.

In Buccal exfoliated cells there were no significant differences between the E and NE groups, neither in respect to the frequency of MNC/1000 cells nor in NBUD/1000 cells. Considering smoking, the SW displayed the lowest NBUD frequencies and the comparison between the SW and NSW showed a significant difference in the frequency of NBUD ($p = 0.0004$, Chi-square test). A positive correlation was found between MNBC/1000 BC in PBLs and MNC/1000 cells ($p = 0.003$, $r = 0.527$, Spearman's correlation) or NBUD/1000 cells ($p = 0.0038$, $r = 0.5199$, Spearman's correlation) in buccal cells in the E group, which was not seen in other groups.

The impact of potential confounding factors was analyzed in respect to each of the effect biomarkers considering the total number of individuals studied, or after stratification by exposure. In this sense, data for each biomarker were separately analyzed according to gender, but no significant differences were observed, in spite women presented a slightly increased mean of SCEs when compared to males (9.33 ± 1.48 *vs.*7.75 ± 1.31, respectively; $p = 0.071$, Mann–Whitney *U*-test) and a slightly lower level of DNA damage as assessed by the percentage of DNA in tail (2.18 ± 0.31 *vs.* 3.38 ± 1.35, respectively; $p = 0.076$, Mann–Whitney U-test), only in the E group. None of the biomarkers was

TABLE 1 | Characteristics of the study population.

Variables	Smoking workers (SW)	Non-smoking workers (NSW)	
		NE	E
Number of workers	19	33	29
Age (years)			
Mean ± SD	39.1 ± 11.1	45.2 ± 12.2[a]	37.2 ± 10.8
Range	18–63	19–66	24–57
Gender (%)			
Female	6 (38.6)	11 (33.3)	4 (13.8)
Male	13 (68.4)	22 (66.7)	25 (86.2)
Smoking habits			
No. of cigarettes per day (Mean ± SD)	16.5 ± 7.5	–	–
Range	3–30	–	–
No. of years of smoking (Mean ± SD)	22.9 ± 10.9	–	–
Range	3–49	–	–
Other ETS exposure* (%)			
Yes	4 (21.1)	3 (9.1)	7 (24.1)
No	12 (63.2)	30 (90.9)	17 (58.6)
No data	3 (15.8)	–	5 (17.2)
Biomarker of exposure (ng/ml)**			
Cotinine concentration (Mean ± SD)	1598.3 ± 806.9	2.2 ± 4.3	7.9 ± 7.3[b]
Range	237.0–3125.0	1–19.0	1–28.0
Working time characterization			
Months in the current job (Mean ± SD)	67.6 ± 54.3	155.6 ± 151.4	74.9 ± 91.2
Range	3–180	2–468	1–408
Hour in a week of service (Mean ± SD)	47.5 ± 12.6	48.9 ± 12.3	39.1 ± 9.7[c]
Range	40–90	14–66	14–70

*[a]Mean NE significantly higher than E (p = 0.008, unpaired t-test); [b]Mean E significantly higher than NE (p = 0.0005, Mann-Whitney U-test); [c]Mean E significantly lower than NE (p < 0.0001) and SW (p < 0.01) with Mann–Whitney U-test; *Exposure outside the workplace in the study, at home; **Methodology described in Pacheco et al. (17); Cotinine average concentrations below the detection limit of the assay were assumed to be 0.1 ng/ml for analysis purpose (quantification limit is 5 ng/ml); SD, standard deviation.*

TABLE 2 | Results of the cytogenetic effect biomarkers.

	Smoking workers (SW)		Non-smoking workers (NSW)				
			NE		E		*p*-value, test (NE *vs.* E)
	n	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	
Lymphocytes							
SCEs	19	9.01 ± 1.64	33	8.06 ± 0.92	29	7.97 ± 1.42	0.472, Mann–Whitney
HFCs	19	14.00 ± 9.48[a]	33	6.79 ± 4.77	29	8.00 ± 7.54	0.892, Mann–Whitney
MNBC/1000 BC	19	6.79 ± 4.09	32	6.22 ± 3.27	29	4.52 ± 2.71[b]	0.004, Chi-square test
CBPI	19	1.56 ± 0.14	32	1.59 ± 0.16	29	1.61 ± 0.16	0.767, Mann–Whitney
Buccal exfoliated cells							
MNC/1000 cells	19	0.48 ± 0.59	32	0.49 ± 0.80	29	0.69 ± 0.90	0.306, Chi-square test
NBUD/1000 cells	19	0.31 ± 0.40[c]	32	0.85 ± 0.83	29	1.1 ± 0.83	0.318, Chi-square test

SD, standard deviation; SCE, sister chromatid exchange; HFC, high frequency cells; MNBC, micronucleated binucleated cells; BC, binucleated cells; CBPI, Cytokinesis-blocked proliferation index; MNC, micronucleated cells; NBUD, nuclear buds; [a]significantly different from NE (p = 0.003; Mann–Whitney U-test) and from E (p = 0.001; Mann–Whitney U-test); [b]significantly different from SW (p = 0.001; Chi-square test); [c]significantly different from both NE and E (p < 0.001, Chi-square test).

impacted by the age of the individuals. Regarding the smoking habits, there was no influence of the number of cigarettes smoked per day or the number of years of smoking in the biomarkers analyzed. Also, no relationship was found between the parameters that characterize the working time and the effect biomarkers. Furthermore, there was no correlation between the exposure biomarker (urinary cotinine concentration) and each of the effect biomarker, when considering the whole group of

TABLE 3 | Results of the comet assay in leukocytes (basal) and of the comet-based challenge assay.

	Smoker workers (SW)		Non-smoker workers (NSW)				
			NE		E		*p*-value
	n	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	(NE *vs.* E)
Basal							
Tail DNA (%)	17	2.94 ± 0.94	32	2.93 ± 0.70	27	3.24 ± 1.34	0.738
Tail length (μm)	17	3.30 ± 1.64	32	3.13 ± 0.80	27	3.00 ± 0.90	0.523
EMS challenge							
Tail DNA (%)	17	35.46 ± 7.48*	32	36.67 ± 10.93	27	26.89 ± 6.95	0.0001
Tail length (μm)	17	35.70 ± 6.83*	32	36.02 ± 8.09	27	29.18 ± 5.90	0.0001

*SD, standard deviation; EMS, Ethyl Methanesulfonate; *Significantly different from E groups ($p < 0.01$, Mann–Whitney U-test).*

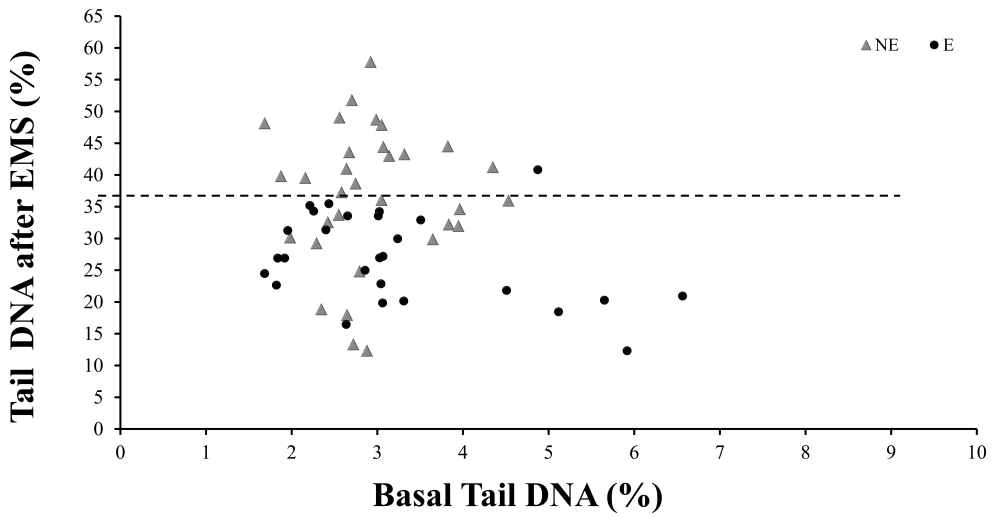

FIGURE 1 | Distribution of the tail DNA from non-exposed (NE) or Exposed (E) workers with and without the *in vitro* challenge with Ethyl Methanesulfonate (EMS). Each data point corresponds to one worker. Dashed line represents the mean value of DNA in tail for the NE, after challenge with EMS.

individuals or after their stratification according to exposure, i.e., E, NE, and SW groups.

Biomarkers of Susceptibility

The distribution of the genotype frequencies relative to the metabolism and DNA repair genes in the studied groups is presented in **Table 4**. The allelic frequencies of $GSTP1^{105}$, $hOGG1^{326}$, $XRCC1^{194}$, $XRCC1^{399}$, $XRCC3^{241}$, NBN^{185}, and $PARP1^{762}$ follow the Hardy-Weinberg conditions ($p < 0.05$, Chi-square test), except the $GSTP1^{105}$genotype on the ETS-exposed group. In the study population, considering the polymorphisms in the $hOGG1^{326}$, $XRCC1^{194}$, and $PARP1^{762}$ genes, the prevalent allele was the common allele (+/+). In addition, when considering the *GSTT1* gene, the wild-type allele was prevalent in the study population; the E group presented a lower prevalence of the null genotype (17.24%) comparatively to the NE (39.39%). For the *GSTM1* gene both genotypes were similarly present (**Table 4**). The distribution of the common and variant alleles between the NE and E groups did not show significant differences for any of the polymorphism analyzed except for NBN^{185} ($p = 0.047$, Fisher exact test), where the E group presented a lower prevalence of the wild-type genotype. No significant differences were observed between SW and E or NE groups for all studied polymorphisms, although the *GSTP1 Ile/Ile* genotype was more prevalent in the E group (62.07%) than in the SW (31.58%).

Influence of Genetic Susceptibility on Effect Biomarkers

To ascertain the potential influence of the metabolism and DNA repair genes genotype in the genotoxic outcomes, the measurements of chromosome and DNA damage in individuals carrying the common or the variant alleles were compared both in the total study population and in each study group (**Tables 5**, **6**). When analyzing the total number of individuals studied, irrespectively of the exposure condition, none of the polymorphisms characterized significantly influenced the level

TABLE 4 | Frequency of metabolism and DNA repair genotypes in the studied groups.

Genes	Genotypes	All (%)	Smoker workers (SW) (%)	Non-smoker workers (NSW)		*p*-value
				NE (%)	E (%)	(NE *vs.* E)
GSTP1^{105}	*Ile/Ile*	41(50.6)	6 (31.58)	17 (51.52)	18 (62.07)	0.679[a]
	Ile/Val	29 (35.8)	11 (57.89)	11 (33.33)	7 (24.14)	
	Val/Val	11 (13.6)	2 (10.53)	5 (15.15)	4 (13.79)	
	*FA**	0.31	0.39	0.32	0.26	
GSTM1	*Present*	41 (50.6)	7 (36.84)	17 (51.52)	17 (58.62)	0.617[b]
	Absent	40 (49.4)	12 (63.16)	16 (48.48)	12 (41.38)	
GSTT1	*Present*	58 (71.6)	14 (73.68)	20 (60.61)	24 (82.76)	0.091[b]
	Absent	23 (28.4)	5 (26.32)	13(39.39)	5 (17.24)	
XRCC1^{194}	*Arg/Arg*	66 (81.5)	18 (94.74)	25 (75.76)	23 (79.31)	0.771[b]
	Arg/Trp	15 (18.5)	1 (5.26)	8 (24.24)	6 (20.69)	
	Trp/Trp	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
	*FA**	0.09	0.03	0.12	0.1	
XRCC1^{399}	*Arg/Arg*	40 (49.4)	10 (52.63)	14 (42.42)	16 (55.17)	0.559[a]
	Arg/Gln	35 (43.2)	9 (47.37)	15 (45.45)	11 (37.93)	
	Gln/Gln	6 (7.4)	0 (0.0)	4 (12.12)	2 (6.90)	
	*FA**	0.29	0.24	0.35	0.26	
XRCC3^{241}	*Thr/Thr*	33 (40.7)	10 (52.63)	11 (33.33)	12 (41.38)	0.356[a]
	Thr/Met	36 (44.4)	8 (42.11)	14 (42.42)	14 (48.28)	
	Met/Met	12 (14.8)	1 (5.26)	8 (24.24)	3 (10.34)	
	*FA**	0.37	0.26	0.45	0.34	
hOGG1^{326}	*Ser/Ser*	57 (70.4)	12 (63.16)	27 (81.82)	18 (62.07)	0.096[b]
	Ser/Cys	21 (25.9)	7 (36.84)	6 (18.18)	8 (27.59)	
	Cys/Cys	3 (3.7)	0 (0.0)	0 (0.0)	3 (10.34)	
	*FA**	0.17	0.18	0.09	0.24	
NBN^{185}	*Glu/Glu*	40 (49.4)	10 (52.63)	20 (60.61)	10 (34.48)	0.047[b]
	Glu/Gln	33 (40.7)	7 (36.84)	10 (30.30)	16 (55.17)	
	Gln/Gln	8 (9.9)	2 (10.53)	3 (9.09)	3 (10.34)	
	*FA**	0.30	0.29	0.24	0.38	
PARP1^{762}	*Val/Val*	54 (69.2)	12 (70.59)	20 (60.61)	22 (75.86)	0.170[b]
	Val/Ala	21 (26.9)	5 (29.41)	11 (33.33)	5 (17.24)	
	Ala/Ala	3 (3.9)	0 (0.0)	2 (6.06)	1 (3.45)	
	*FA**	0.17	0.15	0.23	0.13	

**FA - Frequency of the variant allele for each polymorphism, in the total population and in each studied group. The frequency of the variant allele was calculated considering the heterozygous plus homozygous individuals having the variant allele; [a]Statistical analysis using Pearson Chi-Square (2-sided); [b]Statistical analysis using Fisher exact test (2-sided).*

of the effect biomarkers ($p > 0.05$, Mann-Whitney) (data not shown).

Considering the *GSTP1*, *GSTM1,* and *GSTT1* polymorphisms, no significant differences amongst the possible genotypes were observed, within SW, NE or E groups, for the frequencies of SCEs, MNBC or DNA strand breaks (% tail DNA). It may be noted that, in the SW group, the frequencies of SCEs, HFCs (data not shown) and MNBC were higher in the subjects with the *GSTP1* variant allele (only two individuals), comparatively to those of the WT or heterozygous carriers. The E group maintained the overall trend of lower MNBC comparatively to both SW and NE group which was not influenced by the genotype. On the other hand, SW individuals with the *GSTM1* allele present, showed significantly increased MNBC when compared to E group, while *GSTT1* wild-type individuals from NE group presented increased MNBC when compared to E group ($P = 0.015$, Mann-Whitney *U*-test). In respect to the EMS-induced DNA damage, irrespective of the genotype, the already observed lower levels of DNA damage in the E group comparatively with the NE or SW group is maintained either for the *GSTP1*, *GSTM1,* or *GSTT1*. However, workers' stratification according to their genotype, lead to a lower statistical power, due to the small samples size. This is reflected in the *GSTP1* and *GSTM1*, where for *GSTP1* the difference between the ETS-exposed and NE groups only became significant for the subset of the WT allele carriers whereas for the *GSTM1* polymorphism significance was detected for the comparison between the null allele carriers. Interestingly, in the case of SW, in the absence of the *GSTT1*, a lower level of DNA damage was observed comparatively to *GSTT1* wild-type individuals,

TABLE 5 | Mean (± SD) frequencies of SCEs and MNBC in PBL in the studied groups stratified by genotypes.

Genes	Genotypes	SCEs				MNBC			
		SW	NSW		*p*-value[2]	SW	NSW		*p*-value[2]
			NE	E	(NE *vs.* E)		NE	E	(NE *vs.* E)
GSTP1^{105}	*Ile/Ile*	8.87 ± 1.26	8.29 ± 0.94	7.87 ± 1.08	0.222	4.50 ± 2.35	6.29 ± 2.93	4.94 ± 3.32	0.139
	Ile/Val	8.53 ± 1.38	8.03 ± 0.70	8.25 ± 1.75	0.821	6.91 ± 3.96	6.60 ± 4.50	3.86 ± 1.07	0.257
	Val/Val	12.02 ± 0.85	7.38 ± 1.12	7.92 ± 2.38	0.806	13.0 ± 2.82	6.22 ± 3.27	3.75 ± 0.96	0.076
	p-value[1]	0.064	0.158	0.996		0.072	0.799	0.674	
GSTM1	*Present*	9.20 ± 2.00	8.14 ± 0.77	7.69 ± 1.34	0.241	8.14 ± 3.24[a]	6.50 ± 3.56	4.65 ± 3.40	0.053
	Absent	8.89 ± 1.48	7.98 ± 1.09	8.37 ± 1.45	0.763	6.00 ± 4.45	5.94 ± 3.04	4.33 ± 1.37	0.091
	p-value[2]	0.933	0.679	0.223		0.097	0.879	0.445	
GSTT1	*Present*	8.92 ± 1.63	8.11 ± 0.86	7.92 ± 1.54	0.346	6.60 ± 2.70	7.05 ± 3.56	4.63 ± 2.84	0.015
	Absent	9.25 ± 1.84	7.98 ± 1.05	8.22 ± 0.57	0.693	6.86 ± 4.57	5.00 ± 2.45	4.00 ± 2.12	0.481
	p-value[2]	0.711	0.754	0.386		0.963	0.096	0.907	
XRCC1^{194}	*Arg/Arg*	9.10 ± 1.64[a]	7.97 ± 0.99	7.90 ± 1.47	0.536	7.11 ± 3.95[a]	6.32 ± 3.31	4.91 ± 2.81	0.084
	Arg/Trp	7.38	8.35 ± 0.63	8.23 ± 1.28	0.948	1.00	5.86 ± 3.34	3.00 ± 1.67	0.080
	p-value[2]	–	0.208	0.518		–	0.800	0.107	
XRCC1^{399}	*Arg/Arg*	8.87 ± 1.34	8.00 ± 0.96	7.88 ± 1.30	0.574	7.10 ± 4.04[a]	5.79 ± 3.22	3.75 ± 1.48	0.080
	Arg/Gln + Gln/Gln	9.15 ± 1.99	8.11 ± 0.92	8.07 ± 1.59	0.759	6.44 ± 4.36	6.56 ± 3.87	5.45 ± 3.55	0.250
	p-value[2]	0.806	0.610	0.843		0.593	0.502	0.189	
XRCC3^{241}	*Thr/Thr*	8.69 ± 1.50	8.23 ± 0.58	8.13 ± 1.7 1	0.558	6.80 ± 4.37	6.50 ± 3.21	4.75 ± 2.01	0.141
	Thr/Met + Met/Met	9.36 ± 1.80	7.98 ± 1.06	7.85 ± 1.21	0.671	6.78 ± 4.02[a]	6.09 ± 3.37	4.35 ± 3.16	0.041
	p-value[2]	0.414	0.349	0.690		0.773	0.622	0.252	
hOGG1^{326}	*Ser/ser*	9.21 ± 1.46[b]	8.07 ± 0.95	8.32 ± 1.53	0.694	6.83 ± 4.13	6.59 ± 3.31	4.83 ± 2.83	0.024
	Ser/Cys + Cys/Cys	8.65 ± 1.98	8.01 ± 0.88	7.40 ± 1.03	0.174	6.71 ± 4.35	4.20 ± 2.39	4.00 ± 2.53	0.818
	p-value[2]	0.176	0.907	0.087		0.966	0.142	0.230	
NBN^{185}	*Glu/Glu*	8.75 ± 1.62	8.20 ± 0.76	8.22 ± 1.01	0.660	5.70 ± 2.91	5.79 ± 3.41	3.70 ± 1.83	0.116
	Glu/Gln	9.21 ± 1.82[a]	7.82 ± 1.11	7.55 ± 1.46	0.580	8.43 ± 5.68	6.50 ± 3.34	5.19 ± 3.25	0.221
	Gln/Gln	9.57 ± 1.82	7.93 ± 1.44	9.35 ± 1.73	0.275	6.50 ± 0.71	8.00 ± 2.00	3.67 ± 0.58	0.046
	p-value[1]	0.697	0.399	0.184		0.623	0.364	0.405	
PARP1^{762}	*Val/Val*	9.31 ± 1.76	8.03 ± 1.10	7.95 ± 1.36	0.571	7.42 ± 4.36	6.15 ± 3.23	5.09 ± 2.79	0.147
	Val/Ala + Ala/Ala	8.73 ± 1.61	8.11 ± 0.60	8.03 ± 1.85	0.335	5.00 ± 2.74	6.33 ± 3.47	3.00 ± 1.27	0.052
	p-value[2]	0.527	0.854	0.654		0.365	0.922	0.041	

[1] *Statistical analysis using Kruskal–Wallis;* [2] *Statistical analysis using Mann–Whitney U-test;* [a] *significantly different from E group, within same genotype ($p < 0.05$, Mann-Whitney U-test);* [b] *significantly different from NE group, within same genotype ($p < 0.05$, Mann–Whitney U-test).*

an effect that was opposite of the observed in both E and NE groups.

For the *XRCC1*^{194}, *XRCC1*^{399} and *XRCC3*^{241}, *hOGG1*^{326} and *NBN* ^{185} polymorphisms, no significant influence of the genotype was observed on the frequencies of SCEs, HFCs (data not shown), MNBC or tail DNA, within NE, E, or SW groups. In respect to *PARP1*^{762} polymorphism, a significant difference in the frequency of MNBC was observed within the E group ($p = 0.041$), with the variant allele carriers showing a lower frequency of micronucleated cells than the WT ones. Overall, the E group maintained the trend of lower MNBC and EMS-induced DNA damage comparatively to both SW and NE group.

DISCUSSION

Environmental tobacco smoke is a serious public health concern, recognized as one of the most common indoor pollutants worldwide. Many countries have already successfully implemented smoke-free laws for indoor public spaces and workplaces aimed at limiting exposure to ETS. In Portugal, since 2008, a partial smoke-free law is in place, allowing exceptions, as for example in the case of restaurants, bars or discotheques where smoking is allowed in smokers' designated areas if adequate reinforced ventilation systems are implemented. Thus, exposure to ETS still happens in some Portuguese restaurants and bars (16, 17, 19), meaning that workers remain at risk of ETS exposure. Since tobacco smoke contains a great variety of genotoxic/carcinogenic agents, this study aimed at characterizing the local and systemic biomarkers of genotoxic effects associated to occupational ETS exposure in a set of Lisbon restaurants and bars and the potential influence of genetic polymorphisms on those biomarkers. The quantification of employee's exposure to ETS and the self-reported smoking status was confirmed through urinary cotinine measurement (17).

TABLE 6 | Mean (± SD) values for basal and EMS tail DNA in the studied groups stratified by genotypes.

Genes	Genotypes	Basal tail DNA (%)				EMS tail DNA (%)			
		SW	NSW		*p*-value[2]	SW	NSW		*p*-value[2]
			NE	E	(NE *vs.* E)		NE	E	(NE *vs.* E)
GSTP1[105]	*Ile/Ile*	3.00 ± 0.43	2.86 ± 0.68	3.56 ± 1.44	0.163	35.20 ± 8.31	39.05 ± 10.14	26.65 ± 7.37	0.000
	Ile/Val	3.15 ± 1.06[a]	3.27 ± 0.75	2.26 ± 0.47	0.017	34.28 ± 7.23	35.11 ± 12.64	27.28 ± 6.25	0.104
	Val/Val	1.78 ± 0.13	2.53 ± 0.37	3.36 ± 1.23	0.142	42.06 ± 6.87	31.71 ± 9.76	27.30 ± 7.88	0.327
	p-value[1]	0.200	0.055	0.063		0.386	0.969	0.802	
GSTM1	*Present*	2.60 ± 0.88	2.80 ± 0.62	3.53 ± 1.45	0.132	34.61 ± 7.10[a]	34.29 ± 13.69	26.34 ± 7.40	0.09
	Absent	3.13 ± 0.96	3.07 ± 0.76	2.83 ± 1.08	0.236	35.93 ± 7.98[a]	39.05 ± 6.897	27.68 ± 6.50	0.001
	p-value[2]	0.763	0.407	0.103		0.763	0.522	0.622	
GSTT1	*Present*	3.15 ± 0.95	3.08 ± 0.64	3.27 ± 1.44	0.734	37.60 ± 7.53[a]	34.56 ± 11.91	26.19 ± 7.06	0.010
	Absent	2.45 ± 0.79	2.71 ± 0.74	3.12 ± 0.84	0.349	30.35 ± 4.62[b]	39.75 ± 8.88	29.96 ± 6.11	0.016
	p-value[2]	0.171	0.186	0.851		0.073	0.140	0.236	
XRCC1[194]	Arg/Arg	2.94 ± 0.94	2.90 ± 0.66	3.29 ± 1.37	0.609	35.56 ± 7.48[a]	37.18 ± 10.89	26.84 ± 7.25	0.001
	Arg/Trp	–	3.05 ± 0.87	3.05 ± 1.28	0.808	–	34.87 ± 11.77	27.10 ± 6.13	0.123
	p-value[2]	–	0.837	0.618		–	0.665	0.950	
XRCC1[399]	*Arg/Arg*	3.12 ± 0.28	2.95 ± 0.51	3.21 ± 1.03	0.533	35.58 ± 7.69[a]	37.32 ± 12.64	26.30 ± 6.23	0.011
	Arg/Gln + Gln/Gln	2.79 ± 1.28	2.92 ± 0.83	3.29 ± 1.74	0.893	35.37 ± 7.75	36.17 ± 9.75	27.74 ± 8.11	0.015
	p-value[2]	0.290	0.849	0.622		0.847	0.820	0.693	
XRCC3[241]	*Thr/Thr*	2.91 ± 0.69	3.03 ± 0.69	3.13 ± 1.17	0.725	34.30 ± 6.04	40.13 ± 11.88	28.82 ± 7.24	0.006
	Thr/Met + Met/Met	2.98 ± 1.16	2.89 ± 0.71	3.32±1.46	0.515	36.50 ± 8.80[a]	35.10 ± 10.38	25.56 ± 6.64	0.004
	p-value[2]	0.441	0.684	0.805		0.770	0.155	0.278	
hOGG1[326]	Ser/ser	3.15 ± 1.01	2.88 ± 0.67	3.32 ± 1.43	0.66	33.93 ± 8,06	35.97 ± 11.17	28.56 ± 6.35	0.007
	Ser/Cys + Cys/Cys	2.66 ± 0.80	3.24 ± 0.84	3.09 ± 1.18	0.641	37.66 ± 6.50[a]	40.47 ± 9.72	23.55 ± 7.23	0.014
	p-value[2]	0.696	0.364	0.959		0.283	0.517	0.072	
NBN[185]	*Glu/Glu*	3.09 ± 1.19	2.98 ± 0.84	2.79 ± 1.19	0.337	34.83 ± 6.52	34.83 ± 11.2 4	28.00 ± 6.50	0.058
	Glu/Gln	2.84 ± 0.54	2.92 ± 0.48	3.65 ± 1.43	0.222	38.06 ± 9.18[a]	37.56 ± 9.98	25.41 ± 7.54	0.008
	Gln/Gln	2.60 ± 0.86	2.93 ± 0.70	2.57 ± 0.43	0.513	30.53 ± 6.39	45.35 ± 10.93	30.95 ± 3.58	0.050
	p-value[1]	0.796	0.796	0.163		0.454	0.388	0.290	
PARP1[762]	*Val/Val*	2.91 ± 0.61	2.85 ± 0.67	3.26 ± 1.34	0.45	36.29 ± 7.30[a]	37.83 ± 10.44	26.13 ± 7.05	0.000
	Val/Ala + Ala/Ala	3.07 ± 2.23	3.07 ± 0.75	3.15 ± 1.46	0.527	37.61 ± 8.75	34.73 ± 11.93	30.22 ± 5.95	0.399
	p-value[2]	0.564	0.613	0.755		0.773	0.276	0.190	

[1]Statistical analysis using Kruskal–Wallis; [2]Statistical analysis using Mann–Whitney U-test; [a]significantly different from E group, within same genotype ($p < 0.05$, Mann-Whitney U-test); [b]significantly different from NE group, within same genotype ($p < 0.05$, Mann–Whitney U-test).

In the present study, no effect could be ascribed to ETS exposure in relation to the basal level of DNA damage, as assessed by the comet assay in leukocytes. A similar negative result was obtained when comparing smokers to non-smokers, irrespectively of the ETS exposure. The effect of smoking on DNA damage has been thoroughly studied, mainly as a confounding factor in biomonitoring studies addressing exposure to other compounds (68, 69). Despite that, discrepant results have been reported in the literature, either describing a lack of association between smoking and DNA damage induction (the majority of studies), as reviewed elsewhere (68–70) or an increased DNA damage in smokers comparatively to non-smokers (37, 38, 71–74). Very few studies have been published in respect to the effect of ETS exposure on this biomarker and contradictory results have been reported. In accordance with our results, in peripheral blood lymphocytes of active and involuntary smoking pregnant women, no significant difference was observed between involuntary smokers and non-smokers, but smoking mothers exhibited a statistically significant increase in DNA damage comparatively to involuntary smokers (39). Moreover, newborns displayed results similar to those found for their mothers (39). An increase in DNA damage was reported in lymphocytes of white-collar involuntary smokers and smokers at workplace, comparatively to never smokers, although the mean value obtained for involuntary smokers was similar to ours (38). A similar observation was described in another study, in workers from an elevator manufacturing factory in China, potentially exposed to benzene, were passive smoking at home, but not at the workplace, was significantly associated with DNA damage (37). In children, a significant increase in DNA damage has also been reported after exposure to ETS (41–43). No influence of age or gender in the DNA damage was observed in our study either in the total population or in the studied groups, although women presented a slightly lower level of DNA damage,

as assessed by the percentage of DNA in tail. The impact of age on DNA damage is a matter of controversy, with either positive or negative findings reported, which might depend of different factors such as life-style, descriptors or statistics used, as recently discussed (68). Regarding gender, the overall studies have demonstrated no or equivocal difference between men and women (68). Nevertheless, a study on the impact of ETS exposure in non-smoking workers from casinos and bars in Las Vegas, DNA-damage was significantly increased in a dose-dependent manner with ETS exposure in non-smoker men but not in women (10), in agreement with our finding.

Challenging lymphocytes *ex vivo* with a genotoxicant (e.g., EMS) and measuring induced primary DNA lesions with the comet assay is a functional assay that allows an indirect measurement of the DNA repair competence, which is critical to prevent permanent genetic instability (75, 76). In fact, it is well-known that abnormal DNA repair is a major cause and is mechanistically involved in the development of cancer (77, 78). In this study, blood cells exposure to a single EMS dose allowed the identification of a differential responses in the ETS-exposed group, which presented significantly lower levels of DNA damage, comparatively to the NE and to SW groups. This response suggests that leukocytes from involuntary smokers somehow managed better the EMS-induced alkylating lesions, promoting their rapid repair. In the study of Fracasso et al. (38), lymphocytes of active and non-smokers exposed to ETS challenged with an exogenous oxidative agent (i.e. H_2O_2-induced DNA damage) showed that never smokers not exposed to ETS, had the highest rates of repair of H_2O_2-induced breaks and that the lymphocytes of active smokers exhibited a consistent repair rate at the two administered doses (100 and 200 μM), slower that never smokers, possible due to the presence of high levels of DNA lesions, hardly or not at all repaired (38). However, the passive non-smokers displayed a reduced DNA repair efficacy comparatively to ex-smokers exposed to ETS or to active smokers. The reason for this distinct effect compared to our results, might be related to the challenge agent used. Vodicka et al. reported a higher irradiation-specific DNA repair rate among highly exposed workers in a rubber tire plant and in styrene-exposed lamination workers compared to unexposed or moderately exposed workers (79, 80). The apparent increase in DNA repair following a genotoxicant's acute exposure may reflect a general activation of the DNA repair machinery (79). A similar effect was observed following exposure to benzene (81). In our study, we hypothesize that cells from ETS-exposed individuals may display an adaptive response after EMS challenging due to the continuous low-level exposure to tobacco smoke that works like the conditioning dose. The concept of adaptive response is based on the observation that exposure of cells to a low conditioning genotoxic insult (e.g., radiation, bleomycin, mitomycin C, ethylnitrosurea) leads to their protection against a subsequent higher (challenge) dose of the same genotoxicant, an effect that may result from the upregulation of DNA repair functions (82). For example, there are several lines of evidence for an increase of O^6-methylguanine-DNA methyltransferase (MGMT) activity in the normal tissue of smokers compared to non-smokers although these data awaits conclusive proofs (83). However, Au et al. using the challenge assay, showed that workers exposed to butadiene, pesticides and styrene, and residents exposed to uranium mining and milling waste were found to have significantly higher induction of chromosome aberrations than the respective matched-controls, supposedly due to impaired DNA repair capacity (77, 84).

Therefore, although in this study ETS exposure did not affect the basal level of DNA damage, there is a suggestion that it modulates the blood cells DNA repair response. It remains to be determined whether increased DNA repair capacity in exposed individuals is truly induced, and if so whether a threshold exists for this induction, and whether long-term exposure could exhaust the induction as suggested by Vodicka et al. (80). Furthermore, these findings suggest that ETS, activates a response mechanism that counteracts the negative effects ETS exerted on DNA and the preliminary investigation to try to identify such mechanisms using a proteomic-based approach is ongoing (19).

No increase in either the frequency of SCEs or HFC was observed in ETS-exposed workers (involuntary smokers), as compared to non-exposed workers. Our finding agrees with the negatives data reported in previous studies in workers exposed to ETS in restaurants (33) and in administrative companies (35). The latter studied 106 adult non-smokers divided into two groups according to whether they experienced high or low levels of exposure to ETS as determined from plasma cotinine levels. Nevertheless, in children, ETS exposure was associated with increased SCE (27). On the other hand, it is recognized that the measurement of SCEs in PBLs is a sensitive biomarker of exposure to cigarette smoke (32, 33, 85–92). The cigarette smoke effect was not observed in our study, mainly constituted by light smokers (amount of daily cigarette consumption: 16.47 $\pm$ 7.25; range 3–30). In that regard, a previous study in healthy individuals, showed no differences between light smokers and non-smokers, while the percentages of HFC were significantly higher in smokers than in non-smokers (86). Other studies also observed an effect of smoking in HFC but not in SCE frequency, although only in 1,3-butadiene-exposed workers (93). In our study, the proportion of HFCs was also significantly higher in SW comparatively to the whole non-smokers group and the same trend was observed after stratification into ETS-exposed and NE workers. HFCs are considered relevant to assess the genotoxicity of human chronic exposure to chemicals and have been identified as long-lived lymphocytes which accumulate persistent damage or as a subpopulation of lymphocytes with an increased sensitivity to chemicals (87, 94). Therefore, analyzing the percentage of HFC, as a "measure of SCE rate" may be more sensitive than the mean of SCE to detect effects due to chemical exposure, such as smoking, when an effect is not clearly detected by differences in mean SCE value (95). Our results concerning the influence of gender agree with published studies showing no association with the SCE frequency (87, 92, 96), although positive findings were also reported showing that women display a higher frequency of SCE comparatively to men (85). Also, no influence of age was observed in agreement with recent studies (85, 92).

Interestingly, a lower frequency of micronucleated cells was found in PBLs of involuntary smokers, comparatively to NE or

to SW groups, whereas NE and SW displayed a similar frequency of MNBC. It is important to refer that the frequencies of MNBC obtained for NE and SW groups were within the range of our historical control values. Although this observation in the E group was unexpected, other studies have reported that light smokers (smoking <20 cigarettes per day) and former smokers displayed slightly reduced MN frequencies in comparison to non-smokers (21, 97). An explanation may rely on the fact that the most damaged cells may not survive the culture period in the CBMN assay or may not be able to divide and thus to express chromosome breaks or loss as MN in cultured lymphocytes (21, 97). Another hypothesis also pointed out by the same authors to justify the lower frequency of MN observed in the PBL of light-medium smokers when compared with non-smokers, is that a few cigarettes per day may stimulate an adaptive response, causing an apparent lowering in the MN frequency, and a continued exposure to mutagens/carcinogens may induce resistance to further DNA damage (21, 97). This might be the case in our study, since the observed lower frequency of MN in involuntary smokers is compatible with the hypothesized increased capacity of these workers to repair DNA strand breaks, following *ex vivo* blood cells exposure to EMS. Taken together, the results from both assays may suggest that continuous and repetitive exposure to low level of ETS stimulates a cell-protective response. However, it must stressed that the long-term health consequences from this continuous stimulation cannot be foreseen. A study addressing the effect of ETS exposure on children showed a 30% increase in the frequency of MN (25), although caution should be taken before making extrapolations to adults, since children show increased sensitivity to toxic substances when compared to adults due to differences in chemicals detoxification and excretion pathways (76, 98, 99). In this study, no induction of MNBC was observed in SW as compared to the total NSW population not stratified by ETS exposure. Although some authors reported an increase in the MN frequency in smokers, our results agree with those from a meta-analysis within the HUMN project, that showed that smokers do not exhibit an overall increased MN frequency when compared to non-smokers, which is normally higher in heavy smokers not occupationally exposed to genotoxic agents (21). This was also recently observed in other study (100). Although the effect of age and gender on the MN levels in lymphocytes is well-established, with women having higher levels of MN than men and with MN levels progressively increasing with age (97, 101, 102), that was not observed in our study, possibly due to the sample size and the relatively young age of the participants, mostly constituted by men.

The analysis of MN and NBUD in buccal cells, as a biomarker of local effect, did not detect differences in the number of MNC and NBUD between the E and NE groups, with both groups presenting MN frequencies similar to the average reported for healthy population (1–3 per 1,000 cells) (101–103). A positive correlation between MN in buccal cells and MN in whole blood lymphocytes was found in our study, but only for the E group. This observation agrees with a recent analysis showing that MN frequencies in exfoliated buccal cells correlate with those analyzed in peripheral lymphocytes and that both are valid biomarkers for increased cancer risk in humans (104, 105). However, a decreased level of NBUD was seen in the SW group comparatively to NSW, which might be related to a higher turnover of the oral mucosa cells of smokers. Neither age or gender influenced the level of MNC or NBUDs in our study. Also, within the HUMN(XL), in an analysis of a database of 5,424 subjects with buccal MN values obtained from 30 laboratories worldwide, no effect of gender was evident, while the trend for age was highly significant (102).

The remaining confounding factors, either related to smoking habits or working time did not influence the effect biomarkers analyzed. Furthermore, there was no correlation between the exposure biomarker (urinary cotinine concentration) and each of the biomarkers of effect, when considering the whole group of individuals or after their stratification according to exposure, i.e., E, NE, and SW groups.

Overall, the results of a set of biomarkers of early biological effects showed that ETS did not induce DNA or chromosome damage in blood cells from non-smoking exposed workers. Furthermore, following an *ex vivo* acute genotoxic stimulus, ETS-exposed individuals displayed a higher competence to repair DNA damage than unexposed individuals, which might be related with an adaptive response triggered by the prolonged exposure to a low level of tobacco smoke components.

Genetic susceptibility biomarkers, such as the inherited capacity for xenobiotic biotransformation and DNA damage repair, indicate individual differences that can modulate the response to genotoxic insults (46, 47). Thus, the association of polymorphisms in relevant genes with biological effects following exposure to environmental stressors represents a valuable tool for assessing the individual sensitivity to that exposure, and it may also influence the basal level of DNA or chromosome damage (47, 50). Therefore, in this study, the influence of genetic polymorphisms in genes associated with metabolism and DNA repair on several genotoxicity endpoints was analyzed. For most polymorphisms investigated, no influence was detected in relation to any of the genotoxicity biomarkers within workers from each of the studied groups, possibly due to small sample size. The E group maintained the overall trend of lower MNBC and EMS-induced DNA damage, comparatively to both SW and NE group, which was not influenced by the genotype. The only exception was $PARP1^{762}$ polymorphism. PARP1 is an enzyme involved in the cellular response mechanisms to DNA damage. It participates in DNA base excision repair, single- and double-strand break repair pathways, that are active in the prevention of deletions/insertions induced by alkylating agents. *PARP1* polymorphism *Val762Ala* (rs1136410 T>C) may be associated with prostate cancer (106), esophageal squamous cell carcinoma (107), and breast cancer (108). Recent meta-analyses found a borderline significant association between *PARP1 Val762Ala* polymorphism and overall cancer risk, although after stratification by cancer types, the polymorphism could predispose to gastric cancer, thyroid cancer and cervical cancer, in an Asian population, but not in Caucasian and African populations (109, 110). Involuntary smoking was associated with an increased risk of breast cancer among both pre- and postmenopausal women, depending on the genotype of $PARP1^{762}$ (111), while *Ala762Ala* (rs1136410 C/C) genotype

was associated with an elevated risk of esophageal squamous cell carcinoma in smokers compared to T/T or T/C genotype (107, 112). In the present study, the *PARP1*^{762} homozygous wild-type genotype modulated the MNBC frequency, which was lower in variant carriers, within the E group. Considering the above-mentioned studies, it would be expected that the variant allele would rather be associated with increased MNBC frequency. When looking at EMS-induced DNA damage, the *PARP1*^{762} variant allele carriers presented an increase of DNA damage in E group comparatively to the wild-type carriers, although not significant, due to small number of variant allele carriers. It can be suggested that the partial suppression of PARP1 activity in the variant carriers might have led to an overload of genetic damage in cells, triggering cell death mechanisms and removal of most damaged cell, thereby masking a genotoxic effect. Besides, a decreased MNCB frequency was observed in the variant allele carriers from E group compared to NE, similarly to the slight effect observed in E workers with normal allele, thus suggesting a possible interaction between tobacco smoke exposure and the genotype, in E group, in the genotoxic outcome observed. According to recent meta-analyses, *GSTM1* and *GSTT1* deletion as been associated with lung cancer in overall population (113, 114), while no correlation was observed concerning *GSTP1*^{105}, in overall population (115). Concerning DNA repair-genes, based on meta-analysis, *XRCC1*^{399} and *XRCC1*^{194} polymorphism were significantly associated with lung cancer risk in caucasions (116), and *NBS1*^{195} in Asians, but not caucasions (117). No relation with lung cancer was observed either in *XRCC3*^{241} and *hOGG1*^{326} genotype subjects (118–120). Overall, despite the influence of the studied polymorphisms in the genotoxic biomarkers was not sharp when considering the exposure groups stratification, some influence appears to exist that needs however, to be further investigated in larger groups of workers.

In the present study the results of genotoxicity biomarkers used to assess the early biological effects of ETS in non-smoking workers are presented. The most relevant effect detected in restaurant workers exposed to indoor tobacco smoke was a modified response to a genotoxic challenge, compatible with an adaptive response. It remains to be determined, however, whether the induction of this kind of response may have long term consequences to the health of those workers. The other effects biomarkers characterized in lymphocytes or oral mucosa cells did not detect significant changes in ETS-exposed comparatively to unexposed workers. Although the contribution of individual susceptibility to the outcomes of this exposure did not generate conclusive results, it deserves further attention. Further investigation is also needed to better understand the mechanisms underlying the possible adaptive response reported in this work and its implication to human health. Ongoing work will try to address the relationship between ETS exposure, biomarkers of effect and alterations of the proteome.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comissão de ética do Instituto Nacional de Saúde Doutor Ricardo Jorge. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NV, SA, and HL carried out the experiments and drafted the manuscript. MS and HL conceived the experimental strategy, supervised the work, participated in the data analysis and discussion, and in the manuscript writing and revision. TS and DP were the project coordinators and implemented the study logistics. FV was involved in workers recruitment, contextual data, and samples collection. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.674142/full#supplementary-material

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Integrated Analysis of mRNA- and miRNA-Seq in the Ovary of Rare Minnow *Gobiocypris rarus* in Response to 17α-Methyltestosterone

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17α-Methyltestosterone (MT) is a synthetic androgen. The objective of this study was to explore the effects of exogenous MT on the growth and gonadal development of female rare minnow *Gobiocypris rarus.* Female *G. rarus* groups were exposed to 25–100 ng/L of MT for 7 days. After exposure for 7 days, the total weight and body length were significantly decreased in the 50-ng/L MT groups. The major oocytes in the ovaries of the control group were vitellogenic oocytes (Voc) and cortical alveolus stage oocytes (Coc). In the MT exposure groups, some fish had mature ovaries with a relatively lower proportion of mature oocytes, and the diameter of the perinucleolar oocytes (Poc) was decreased compared with those of the control group. Ovarian VTG, FSH, LH, 11-KT, E2, and T were significantly increased after exposure to 50 ng/L of MT for 7 days. Unigenes (73,449), 24 known mature microRNAs (miRNAs), and 897 novel miRNAs in the gonads of *G. rarus* were found using high-throughput sequencing. Six mature miRNAs (miR-19, miR-183, miR-203, miR-204, miR-205, and miR-96) as well as six differentially expressed genes (*fabp3*, *mfap4*, *abca1*, *foxo3*, *tgfb1*, and *zfp36l1*) that may be associated with ovarian development and innate immune response were assayed using qPCR. Furthermore, the miR-183 cluster and miR-203 were differentially expressed in MT-exposed ovaries of the different *G. rarus* groups. This study provides some information about the role of miRNA–mRNA pairs in the regulation of ovarian development and innate immune system, which will facilitate future studies of the miRNA–RNA-associated regulation of teleost reproduction.

Keywords: 17α-methyltestosterone, *Gobiocypris rarus*, RNA-seq, miRNA-seq, ovary

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INTRODUCTION

17α-Methyltestosterone (MT) is a synthetic organic compound, which is a typical endocrine disruptor widely available in the environment. MT is commonly found in sewage from paper mill and domestic and livestock manure, especially in aquatic reproduction; approximately 1.33 ng/L of MT was detected in the sewage discharge of chemical plants, while 4.1–7.0 ng/L MT was detected in Beijing wastewater samples. MT can inhibit the activity of steroidogenic enzymes, especially

aromatase (Kortner and Arukwe, 2007), and cause alterations in sex steroid hormone levels in the body (Lyu et al., 2019; Meng et al., 2019). MT exposure can, thus, lead to sex reversal in many kinds of fish, such as zebrafish (Lee et al., 2017) and orange-spotted grouper (Wang et al., 2018; Huang et al., 2019; Lyu et al., 2019). When 7-month-old Pengze *Carassius auratus* were exposed to MT (50 and 100 μg/L) in semistill water, they exhibited ovaries that were degenerated and atretic in both groups. Moreover, MT impaired the gonad development of *C. auratus* (Zheng et al., 2016) and the rare minnow species *Gobiocypris rarus* (25–100 ng/L) (Gao et al., 2014, 2015; Liu S. et al., 2014; Liu Y. et al., 2014; Liang and Zha, 2016). MT (302.5 ng/L) disturbed the gene expression of the hypothalamus–pituitary–gonadal axis in mummichog (*Fundulus heteroclitus*) (Rutherford et al., 2019). However, most studies, so far, have focused on gene expression without referring to the underlying microRNA (miRNA) regulation.

miRNAs are short (21–23 nucleotides), single-stranded, non-coding RNAs that form complimentary basepairs with the 3′ untranslated region of target mRNAs within the RNA-induced silencing complex (RISC) and block translation and/or stimulate mRNA transcript degradation (Kelly et al., 2013; Stavast and Erkeland, 2019). The total set of transcripts (mRNA and non-coding RNA) involved in the transcriptome is transcribed at a specific organization during a particular developmental stage (Mardis, 2008). In an organism, a single miRNA may control the expression of several genes, or multiple miRNAs work simultaneously to control the expression of a single gene (Bartel, 2004). Previous studies showed that miRNA may be an inducible factor to increase the complexity of organism with their roles in regulating gene expression (Heimberg et al., 2008). Recent studies have confirmed that miRNAs play an important role in regulating the development of fish embryos and hypoxia responses in the liver of darkbarbel catfish (Zhang et al., 2016), sex determination and differentiation in the gonad tissue of dark sleeper (Zhao et al., 2017), and fundamental cellular processes in the gut and liver of zebrafish (Renaud et al., 2018). In addition, miRNAs negatively regulate the function of genes associated with reproduction. For example, Bizuayehu et al. (2019) found that five biased miRNAs, ssa-let-7a, ssa-miR-10a, ssa-miR-20a, ssa-miR-130a, and ssa-miR-202, were related to the egg quality of Atlantic salmon (*Salmo salar*), while Fischer et al. (2019) found that FoxH1 repressed miR-430 in gonad development of zebrafish. Moreover, miRNAs dre-miR-143, dre-miR-101a, dre-miR-202-5p, dre-let-7c-5p, and dre-miR-181a-5p are related to gonad development of *Trachinotus ovatus* (He et al., 2019); ccr-miR-24, ccr-miR-146a, ccr-miR-192, ccr-miR-21, ccr-miR-143, and ccrmiR-454b regulate the gonad development of common carp (*Cyprinus carpio*) exposed to atrazine (Wang F. et al., 2019); and miRNA-26a/cyp19a1a regulates feedback loop in the protogynous hermaphroditic fish, *Epinephelus coioides* (Yu et al., 2019). Some recent studies have also found negative regulators (*let-7a/b/d*) of the ovary development process, in blunt snout bream (Lan et al., 2019), as well as those involved in steroid hormone synthesis related pathways, through miRNA–mRNA analysis in Japanese flounder (Zhang et al., 2019). Atrazine can upregulate aromatase expression through miRNAs, which supports the hypothesis that atrazine has endocrine-disrupting activity by altering the gene expression profile of the HPG axis through its corresponding miRNAs (Wang G. et al., 2019).

Some proteins, such as β-catenin in freshwater mussel *Hyriopsis cumingii* (Wang F. et al., 2019) and vitellogenin (VTG) in *Nothobranchius guentheri* (Liu et al., 2017), may participate in a variety of physiological activities like immune regulation and sex determination. BPA can increase the mRNA stability of β-*catenin* via suppressing the expression of miR-214-3p, which can directly target the 3′UTR of β-*catenin* mRNA (Zeng, 2020). Liu et al. (2021) found that the expression of *vtg* was regulated directly by miR-34, and the expression level of *vtg* in the agomiR-9c/-263a group was significantly decreased, while that in the antagomiR-9c/263a group, it was significantly increased. Such results indicate that miR-9c and miR-263a could regulate *vtg* indirectly by inhibiting the expression of *cyclins* and *CDKs*, thus, affecting the development of the ovary. Meanwhile, the changes in *vtg* expression are more intuitive to manifest that these genes can affect ovarian development through the regulation of miRNAs.

In this study, we tested the sex hormone (E2, T, FSH, LH, and 11-KT) and VTG of *G. rarus* exposed to MT (25, 50, and 100 ng/L) for 7 days. At the same time, we monitored whether there was a morphological change in the ovaries in rare minnow related to different concentrations of MT, thereby, investigating the differentiating effects of different concentrations of MT on ovarian development and follicle maturation in *G. rarus*. In this study, we aim to explore the negative regulatory effect of miRNA on mRNA expression after MT administration in order to find miRNA–target gene pairs. In addition, we will further study miRNA–mRNA interaction networks, which may help explore the underlying mechanisms of the reproduction and immune system of *G. rarus*.

MATERIALS AND METHODS

Experimental Animals

All experiments for animals were approved by the Institutional Animal Care and Use Committee of Shanxi Agriculture University, and the IACUC No. is SXAU-EAW-2018G.R.0201. As MT is insoluble in water and only soluble in organic solvents, the MT stock solution was prepared in anhydrous ethanol. The experimental group of *G. rarus* from the same family was selected through artificial fertilization, and the females were segregated after sexual maturity according to distance between the hind fin and the tail fin because such distance of female fishes is longer than that of the male. In cases when it was difficult to distinguish sex by using external appearance, the ovaries of fishes were observed after the exposure experiment. Before the exposure experiment, 6-month-old female *G. rarus* were domesticated for 1 week after grouping. Then they were treated with different concentrations of MT (25, 50, and 100 ng/L named as MT25-F, MT50-F, and MT100-F, respectively, test groups) or with 0.0001% anhydrous ethanol (solvent control group) for 7 days. The concentration and exposure durations were determined according to our reports (Gao et al., 2014, 2015; Liu S. et al., 2014; Liu Y. et al., 2014) and the studies of Liang and Zha (2016)

and Rutherford et al. (2019). All experiments were performed in triplicate for the three treatment and one control groups. A total of 12 aquariums were used, with a volume of 80 L each. There were 25–30 *G. rarus* females in each aquarium, ensuring the ratio of 1 g of fish for every 1 L of water. They were fed regularly and in fixed quantities every day. The method of semistatic water exposure was utilized to change half of the water in the aquarium, while sucking the sewage (residual bait and feces) and simultaneously adding the same amount of water along with the corresponding amount of MT solution, to ensure that the MT concentration in the aquarium remained unchanged.

Measurement and Sampling of Biological Indicators

Three fish were selected from each aquarium and anesthetized. Biological indicators including the total length, body length, and body weight of all fish in the treatment and the control groups were measured (n = 6). The ovaries of six fish in each group (n = 6, a total of 18 fish from three repeated experiments) were halved and fixed with Bouin's solution for ovarian morphological analysis of *G. rarus*. The ovaries were fixed for 24 h, following which, they were dehydrated with alcohol and treated with xylene to make the tissue transparent. The ovarian tissues were embedded in wax blocks. Continuous wax strips of 6 μm were prepared from these blocks using Leica M2245 (Germany, Leica Biosystems). H&E staining was performed, and the images were observed and photographed under an RCH1-NK50I light microscope. We used Image J v1.53a (Publisher Wayne Rasband) to count the number of cells in the H-E slices.

ELISA

According to the methods reported by Lange et al. (2008), the whole body trunk (n = 3 per group) was taken from the severed tails via a capillary tube and quickly transferred to a heparinized centrifuge tube, and then the protease inhibitor (2 trypsin inhibitor units/ml) was added. Centrifugation was carried out at 21,380 × g for 15 min at −20°C. The supernatant was carefully pipetted out and stored at −80°C, for the determination of VTG, follicle-stimulating hormone (FSH), luteinizing hormone (LH), 11-ketotestosterone (11-KT), 17β-estradiol (E2), and testosterone (T) levels. These were determined using an ELISA kit (Nanjing Jiancheng Biotechnology Co., Ltd.). According to the protocols provided by the manufacturer, and for the specific steps, please refer to the instructions of such ELISA kits from Nanjing Jiancheng Biotechnology Co., Ltd. All samples and standards were run in triplicates (both the CV of inter-assay and intra-assay were less than 10%). When the standard curve of VTG, FSH, 11-KT, E2, LH, and T was $R^2 \geq 0.99$, it indicated that the experimental results are reliable.

Samples for the Sequencing

We used trizol one-step method to extract the total RNA of one ovary of each fish, and then we mixed three fish RNA in the same aquarium to prepare a mixed sample, each group of three repeated tests to prepare three mixed samples, treatment group, and control group for a total of 12 samples (n = 12 groups in total with n = 3 per group) for mRNA (n = 3 per group with a total of nine individuals) and miRNA (n = 3 per group with a total of nine individuals) sequencing (Illumina HiSeqTM 2500, Guangzhou Gene *Denovo* Biotechnology Co.).

RNA- and miRNA-Seq

Methods and protocols for total RNA extraction, RNA quantity assessment, library construction, and RNA sequencing were followed as provided in the previous studies (Liu S. et al., 2014; Gao et al., 2015; Renaud et al., 2018). The gene abundances were calculated and normalized to reads per kb per million (RPKM) using the DESeq2 and EBSeq software. "Up_diff" or "down_diff" were classified according to the RPKM values, whereas for miRNA-seq, all of the clean tags were mapped to the reference transcriptome (GenBank Release 209.0, Rfam database 11.0, miRBase database 21.0 for known miRNAs) to identify and remove rRNA, scRNA, snoRNA, snRNA, and tRNA from the miRNAs (Friedländer et al., 2012). The novel miRNA candidates were identified via the Mireap_v0.2 software, and the expression levels of both known and novel miRNAs were calculated and normalized to transcripts per million (TPM). Principal component analysis (PCA) was performed using R package models. MiRNA differential expression based on normalized deep sequencing counts was analyzed by t-test. Comparisons between control and MT-exposed groups were made to identify significant DEMs [$|\log_2(\text{fold change})| > 1$ and $p \leqq 0.05$]. Data normalization followed the procedures as described in a previous study (Cer et al., 2014).

Differentially Expressed Genes and DE miRNAs

We identified DEGs and DEMs with a fold change ≥ 2 and a false discovery rate (FDR) ≤ 0.05 among the comparisons across samples or groups. These common target genes from RNA- and miRNA-seq data were considered for further analysis. Gene Ontology (GO) enrichment and pathway-based analysis were simultaneously provided. RNAhybrid (v2.1.2) + svm_light (v6.01), Miranda (v3.3a), and TargetScan (Version 7.0, Agarwal et al., 2015) were used to predict the target gene–miRNA pairs.

Short time-series expression miner (STEM) was used to finally reveal the expression tendency of DEGs, and weighted gene co-expression network analysis (WGCNA v1.47, Filteau et al., 2013) was adopted to find the co-expression networks that were constructed. This study analyzed and identified the biological function of each miRNA–mRNA pair with a negative correlation.

qPCR to Verify the Selected miRNAs

According to the results of the bioinformatics analysis, miR-19, miR-183, miR-203, miR-204, miR-205, miR-96, and their target genes in the immune process/steroidogenesis pathway were selected for qPCR validation (n = 3 per group). The cDNA was obtained by reverse transcription of total ovarian RNA, and the reverse transcription primers with a stem ring structure and their corresponding PCR primers were designed to detect the miRNA. The miRNA results were compared with the results of qPCR to determine the consistency of the two experimental results and

confirm the miRNAs associated with steroid hormone synthesis and immune system in *G. rarus*. *fabp3* (fatty acid-binding protein 3, related to apoptosis), *mfap4* (microfibrillar-associated protein 4, immune response with bacterial challenge), *abca1* (ATP-binding cassette transporter A1, cholesterol metabolism), *foxo3* (forkhead transcription factor 3a, TGFβ-induced apoptosis and longevity), *tgfb1* (transforming growth factor β1, disease like apoptosis and tumor), and *zfp36l1* (the tristetraprolin or tristetraprolin family of CCCH tandem zinc finger proteins, TNFα target) were selected for qPCR verification. U6snRNA and *ef1a* were used as endogenous controls (primers presented in **Supplementary Table 1**) for the selected miRNAs and RNAs, respectively. Amplification efficiencies of the detected genes ranged from 90 and 110%.

Data Analyses

All data were analyzed by SPSS 19.0 and presented as mean ± SD. One-way ANOVA with Dunn's *post hoc* test was used. Significance levels or *p*-values were stated in each corresponding figure legend. Significance was accepted at the level of $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$). The Levene's test was used to determine the homogeneity of variance (O'Neill and Mathews, 2002).

RESULTS

Morphological Changes

The total weight, total length, and body length measured ($n = 6$ in each group) are listed in **Table 1**. The total weight and body length were significantly decreased in the 50-ng/L MT groups ($p < 0.01$). The major oocytes in the ovaries of the control group were vitellogenic oocytes (Voc) and cortical alveolus stage oocytes (Coc) (**Figure 1a**). In the 25-ng/L MT exposure groups, some fish had mature ovaries with a relatively lower proportion of mature oocytes (**Figure 1b**). In the 50-ng/L MT-treated groups, the Coc and perinucleolar oocytes (Poc) were predominant in the ovaries, and the diameter of the Poc was decreased compared with those of the control group (**Figures 1c,d**).

Sex Steroid Hormone Activity

The levels of sex steroid hormone activity ($n = 3$) in the female rare minnow in response to MT are shown in **Table 1**. Gonadal FSH, LH, 11-KT, and T ($p < 0.01$) were significantly decreased after MT exposure at 25 ng/L for 7 days. Gonadal VTH, FSH, LH, 11-KT, E2, and T were significantly increased after exposure to 50 ng/L of MT ($p < 0.01$) for 7 days. Gonadal LH ($p < 0.05$) and T ($p < 0.01$) were significantly decreased and increased, respectively, following MT exposure at 100 ng/L for 7 days.

RNA- and miRNA-Seq Analysis

In RNA-seq, the gene number and ratio showed no significant changes across different groups ($n = 3$) (**Supplementary Table 2**). For miRNA-seq, total non-coding RNA reads, rRNA, snRNA, snoRNA, tRNA, known miRNA num, novel miRNA num, miRNA number, and target gene number showed no significant changes across different groups ($n = 3$). The significant DEGs for normalized gene expression among different MT groups were identified. In comparison with those of the control group, 5,233 (MT25-F vs. Con-F), 1,663 (MT50-F vs. Con-F), and 1,222 (MT100-F vs. Con-F) genes were detected as significant DEGs. The MT50-F and MT100-F groups presented 3,200 and 1,875 significant DEGs, respectively, compared with those of the MT25-F groups, whereas the MT100-F groups presented 801 significant DEGs compared with those of the MT50-F groups (**Supplementary Table 3**). The results indicated that there were 340 identical DEGs between the MT25-F (Con-F vs. MT25-F) and MT50-F (Con-F vs. MT50-F) groups, 220 identical DEGs between the MT25-F (Con-F vs. MT25-F) and MT100-F (Con-F vs. MT100-F) groups, and 41 identical DEGs between the MT50-F (Con-F vs. MT50-F) and MT100-F (Con-F vs. MT100-F) groups (**Figure 2A**). For miRNA-seq, 76 (MT25-F), 27 (MT50-F), and 73 (MT100-F) DEMs were identified in comparison with those of the control group (**Supplementary Figure 1a**). Within the 76, 27, and 73 DEMs in the 25, 50, and 100-ng/L MT group, 17, 8, 24, and 59, 19, 49 were annotated to known and novel miRNA, respectively. There were three identical DEMs in the MT25-F, MT50-F, and MT100-F groups (**Figure 2B**).

In RNA-seq, several KEGG pathways like cytokine–cytokine receptor interaction, phagosomes, and Jak-STAT signaling pathway were enriched (**Figure 3B**). The top five KEGG pathways, e.g., metabolism (global and overview, lipid, and carbohydrate metabolism as the top three) were enriched (**Figure 3A**), while for miRNA-seq, the top five KEGG pathways were similar to those found in RNA-seq (**Figure 3C**). With respect to RNA-seq, MT concentration explained the largest fraction of the variation (24.8% along PC1, $p < 0.05$; **Supplementary Figure 1b**) after accounting for the variation present. Approximately 11% of the variation was explained by PC2, while 10.1% of the variation was explained by PC3.

TABLE 1 | Biological indicator and the content of hormones in treatment and control groups.

Group	Body length (cm)	Total length (cm)	Total weight	VTG (ng/mg)	FSH (mIU/mg)	11-KT (pg/mg)	E2 (pg/mg)	LH (mIU/mg)	T (pg/mg)
Control	3.62 ± 0.14	4.76 ± 0.17	1.26 ± 0.23	233.52 ± 53.45	57.71 ± 0.47	33.54 ± 1.72	4.25 ± 0.14	15.01 ± 0.23	81.00 ± 0.37
25 ng/L methyltestosterone (MT)	3.56 ± 0.55	4.70 ± 0.52	1.20 ± 0.40	181.19 ± 8.69	46.46 ± 0.32*↓	26.44 ± 0.47*↓	3.74 ± 0.41	11.42 ± 1.03*↓	69.67 ± 0.15**↓
50 ng/L MT	3.00 ± 0.21**↓	4.16 ± 0.34	0.77 ± 0.19**↓	531.09 ± 9.51**↑	112.56 ± 1.88**↑	65.96 ± 0.49**↑	9.24 ± 0.19**↑	23.33 ± 1.43**↑	200.03 ± 2.74**↑
100 ng/L MT	3.30 ± 0.33	1.12 ± 0.25	1.12 ± 0.25	231.41 ± 1.51	60.96 ± 4.22	37.06 ± 1.94	5.04 ± 0.20	11.69 ± 1.21*↓	96.37 ± 0.00**↑

*$p < 0.01$, **$p < 0.05$.

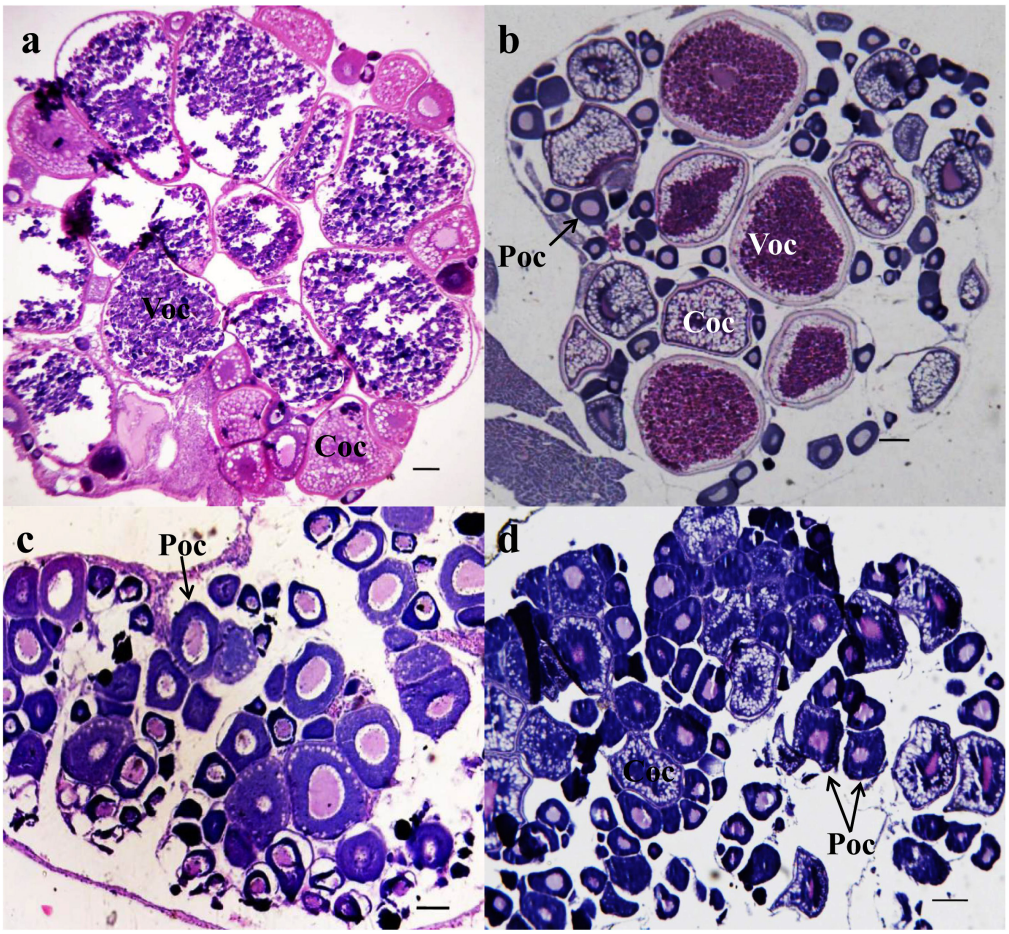

FIGURE 1 | Photomicrographs of transverse ovary sections of adult *Gobiocypris rarus* unexposed and exposed to methyltestosterone (MT) (n = 6). H&E staining, scale bars = 150 μm. **(a)** The 7-day control group ovary, **(b)** 25 ng/L of MT for 7-day exposure, **(c)** 50 ng/L of MT for 7-day exposure, and **(d)** 100 ng/L of MT for 7-day exposure. Voc, mature oocyte; Coc, pre-yolk oocyte; Poc, primary oocyte.

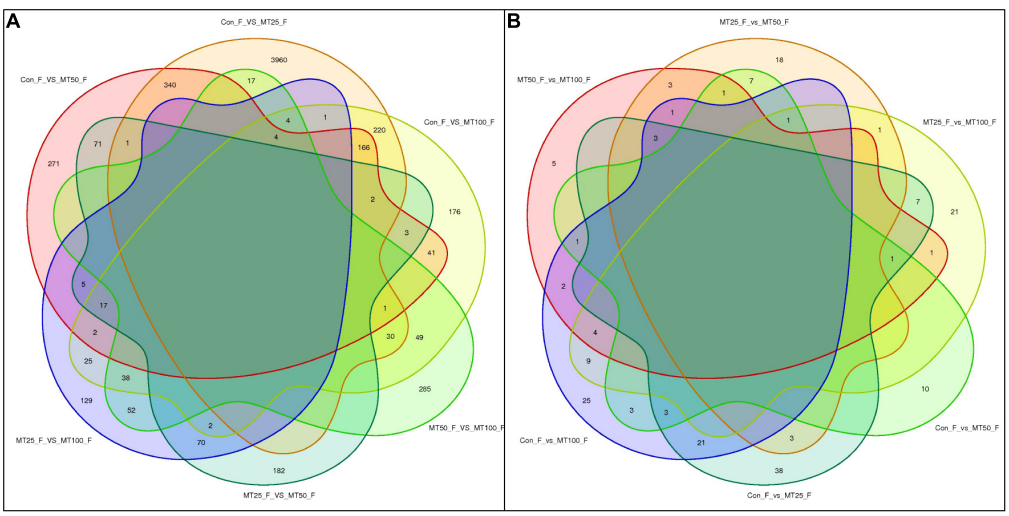

FIGURE 2 | Venn diagram analysis of differentially expressed genes (DEGs). **(A)** DE miRNAs miRNA (DEMs). **(B)** In rare minnow ovaries after MT (25, 50, and 100 ng/L) treatment.

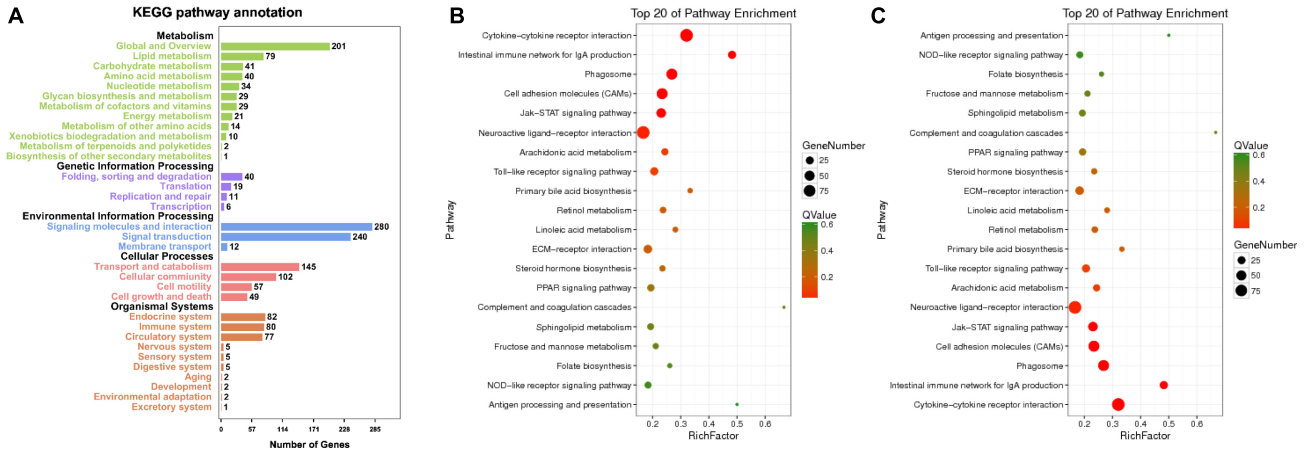

FIGURE 3 | Enriched KEGG pathway annotation of RNA **(A)** and top 20 KEGG pathways of RNA **(B)** and miRNA **(C)**. Statistical summary of distribution of genes in each pathway in each trend. Rich factor refers to the ratio of the number of genes in the pathway entry in the DEGs to the total number of genes in the pathway entry in all genes. The larger the rich factor is, the higher the degree of enrichment is. Q-value is the *p*-value corrected by multiple hypothesis test, and the value range is 0–1. The closer to zero, the more significant the enrichment is. The graph is drawn with the top 20 pathways sorted by Q-value from small to large.

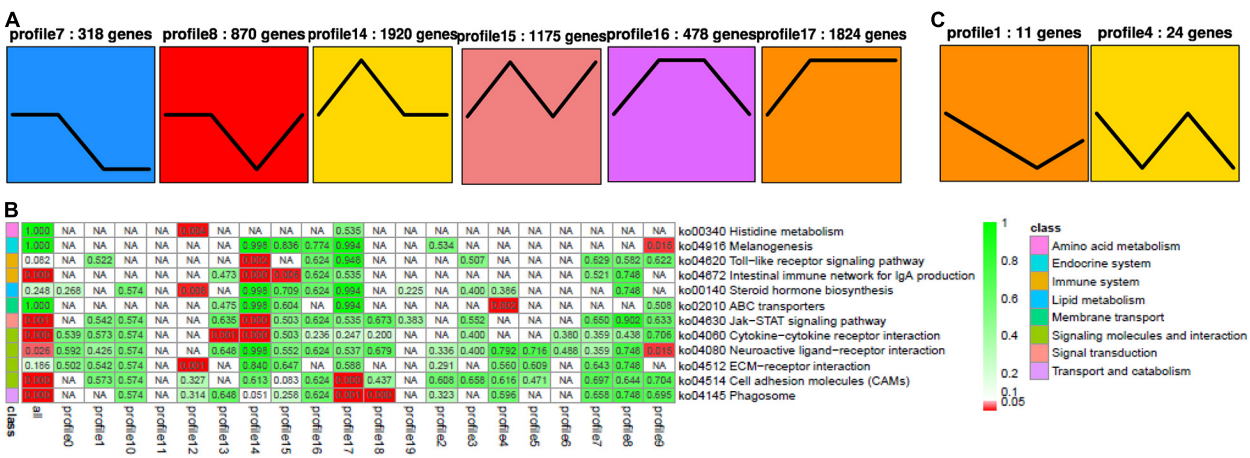

FIGURE 4 | STEM analysis of RNA **(A)** with its relevance **(B)**, and miRNA **(C)**. The ID of the trend and the number of genes in the trend are shown at the top of the figure; the trend block with color ($p < 0.05$): the trend with significant enrichment and the trend block with a similar trend have the same color. In the pathway enrichment analysis *p*-value (Q-value) heat map, the filtering standard is less than or equal to 0.05 in at least one trend. The color bar legend "class" on the left represents the KEGG B class pathway annotation. Each row represents a KEGG pathway annotation, and each column represents a trend or all trends. The number in the grid is the *p*-value/Q-value; "NA" means that the pathway is not enriched in the trend. The darker the lattice color is, the more significant the pathway enrichment is.

In contrast, for miRNA, MT concentration explained the largest fraction of the variation (43.7% along PC1, $p < 0.05$; **Supplementary Figure 1b**) after accounting for the variation present, 18.7% of the variation was explained by PC2, while 11.9% of the variation was explained by PC3.

STEM Analysis

For RNA-seq, 318, 870, 1,920, 1175, 478, and 1,824 DEGs were identified in profiles 7, 8, 14, 15, 16, and 17, respectively, by STEM analysis ($p < 0.05$, **Figure 4A**). All these DEGs were enriched in several pathways (**Figure 4C**), including Toll-like receptor (except for profile 14), IgA production of intestinal immune network (especially for profiles 14 and 15), Jak-STAT (profile 14), cytokine and its receptor interaction (profile 14), neuroactive ligand–receptor interaction (only in profile 9), cell adhesion molecules (CAMs) (profile 17), and phagosomes (profile 17, **Supplementary Table 4**). In miRNA-seq, 11 and 24 DEMs were identified in profiles 1 and 4, respectively ($p < 0.05$, **Figure 4B** and **Supplementary Table 5**).

Among these small RNAs and RNAs, profile 1 of miRNA was most associated with profiles 14 and 17 of RNA, while it was least associated with profile 16. Furthermore, profile 4 of miRNA was most associated with profiles 8, 14, 15, and 17, while it was least associated with profiles 7 and 16. In general, profiles 14 and 17 were the most related, while 16 was the least related. When we looked for miRNA–target gene interaction network

FIGURE 5 | Enriched KEGG pathway annotation for miRNA–mRNA pairs by the weighted gene co-expression network analysis (WGCNA analysis) using Q-value.

using WGCNA analysis, it showed the highest occurrence in the green and turquoise pathways (**Figure 5**). Within the green groups, amino acid and carbohydrate metabolism, endocrine system, global and overview, lipid metabolism and metabolism, etc., were enriched, whereas in the turquoise group, cell growth and death, replication and repair, translation, and endocytosis, etc., were enriched.

In addition, a total of 3,949 miRNA–mRNA pairs with negative correlations were identified; moreover, under the state of profile 1, 940 miRNA–mRNA pairs were also different, and 2,663 miRNA–mRNA pairs of profile 4 were identified. Therefore, when miRNAs are induced by MT, their target mRNAs are downregulated and vice versa.

qPCR Validation of Differentially Expressed Genes

Finally, we chose six negative miRNA–mRNA interactions with six mature miRNAs (miR-19, miR-183, miR-203, miR-204, miR-205, and miR-96) and six validated mRNAs (*fabp3*, *mfap4*, *abca1*, *foxo3*, *tgfb1*, and *zfp36l1*). The sequencing results were consistent with the validation with qPCR (n = 3). *Abp3*, *mfap4*, *abca1*, *foxo3*, *tgfb1*, and *zfp36l1* were significantly increased in the female rare minnow exposed to 25 ng/L of MT. *Mfap4* was significantly increased in the female fish exposed to 50 ng/L of MT. miRNA-19 and miRNA-183 were significantly decreased in the female rare minnow exposed to 25 ng/L of MT. miRNA-96 and miRNA-203 were significantly decreased in the female rare minnow exposed to 50 ng/L of MT. miRNA-183 was significantly increased in the female fish exposed to 50 ng/L of MT. We also found that several genes played critical roles in multiple pathways. For example, *kirrel* and *eef1a1* showed a negative correlation with miR-430 (**Figure 6** and **Supplementary Table 5**).

DISCUSSION

A growing number of studies have shown that ovarian development is suppressed by MT exposure (Gao et al., 2014, 2015; Liu S. et al., 2014; Liu Y. et al., 2014; Rutherford et al., 2015; Zheng et al., 2016). In the present study, we found the decreasing Voc numbers in ovaries, indicating that MT could inhibit the gonadal development of female fish when exposed for 7 days. These results suggested that MT may have more disrupting effects on the ovary at an earlier stage, while a possible stress adaptation occurred in fish that weakened these disrupting effects with the prolongation of the exposure period. A previous study indicated that MT inhibited the E2, LH, FSH, LH, 11-KT, and T in *Pseudorasbora parva* (Wang et al., 2020). In the present study, we found that the biological indicators and hormone levels were significantly altered in the MT-treated female fish compared with the other test groups. FSH, LH, 11-KT, and T were significantly decreased in female fish after MT exposure to 25 ng/L for 7 days. 11-KT plays an important role in controlling pre-Voc growth in *A. japonica* (Lai et al., 2018). It indicates that MT inhibits follicle maturation by inhibiting steroid hormones. In Leydig cells of male rat treated with testosterone for 60 days, the transcriptional downregulation of steroidogenic enzymes

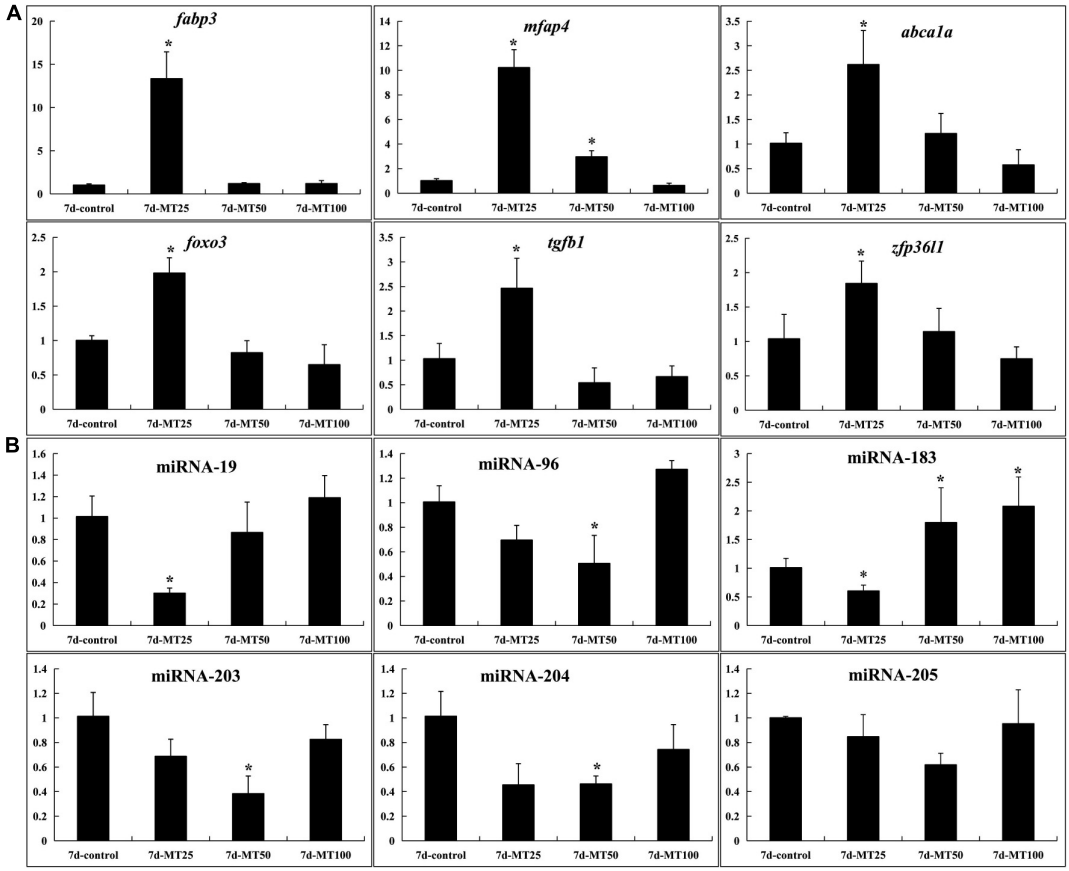

FIGURE 6 | qPCR verification ($n = 3$). *ef1α* and *U6* were used as reference gene for genes and miRNAs, respectively. The data of treatment group and control group were analyzed using the $2^{-\Delta\Delta Ct}$ method. The results were represented as the mean ± SD of female fish. $^{*}p < 0.05$. **(A)** Ovary RNA. **(B)** Ovary miRNA expression. The name of the genes and their potential functions are as follows: *fabp3* (fatty acid-binding protein 3, related to apoptosis), *mfap4* (microfibrillar-associated protein 4, immune response with bacterial challenge), *abca1* (ATP-binding cassette transporter A1, cholesterol metabolism), *foxo3* (forkhead transcription factor 3a, TGFβ-induced apoptosis and longevity), *tgfb1* (transforming growth factor β1, disease like apoptosis and tumor), and *zfp36l1* (the tristetraprolin or tristetraprolin family of CCCH tandem zinc finger proteins, TNFα target).

coupled with significantly decreased LH levels in circulation (Kostic et al., 2011) suggests that MT could regulate androgen production through LH-LHR-cAMP signaling. In the present study, the cause of induced VTG synthesis for MT probably is that MT can be aromatized into methylestradiol (ME2), and ME2 with estrogenic effect subsequently upregulates VTG via the hepatic estrogen receptor (Hornung et al., 2004). The results of our manuscript and previous studies suggest that MT plays roles in the gonadal differentiation and maturation in the rare minnow.

Herein, the involvement of the miRNA–mRNA regulatory network in this phenomenon has not been reported yet. DEGs (924) and DEMs (seven) were identified in this group for mRNA and miRNA, respectively, and these were enriched in metabolic pathways, cytokine–cytokine receptor interaction, etc. The integrated miRNA–mRNA analysis revealed that among those pathways with $p < 0.05$, the latter four pathways were further enriched in the STEM analysis. Six genes (*fabp3*, *mfap4*, *abca1*, *foxo3*, *tgfb1*, and *zfp36l1*) were differentially upregulated in the 25-ng/L MT exposure group. Through RNA sequencing, we successfully identified 924 upregulated and 739 downregulated DEGs in the 50-ng/L MT groups compared with control group, and these were concentrated in profiles 14 and 17. Moreover, from miRNA sequencing, we successfully identified seven upregulated and 20 downregulated miRNAs, which were identified in profiles 1 and 4. Among these, miR-19, miR-183, miR-203, miR-204, miR-205, and miR-96 were downregulated. It has been reported that retinoic acid and *cyp26a1* are necessary only during the early stages of somatogenesis (Sirbu and Duester, 2006), wherein it represses the expression of miR-19 family members as the 3′UTR of *cyp26a1* as a *bona fide* target of miR-19, which was identified using *in vivo* reporter analysis (Franzosa et al., 2013). The present study showed that the targeted genes of miR-19, *fabp3* and *tgfb1*, were upregulated following MT exposure.

In another study, miR-19 was reported to directly target the TGFβ pathway associated with the inflammation pathway (Li et al., 2012), whereas the miR-130a–*fabp3* pair was reported to play a vital role in the PI3K/AKT–mTOR pathway (Chen et al., 2018). Recent studies reported that miR-96 was important in otic vesicle development, involved in the hearing process, along with

miR-183 (target *mfap4*) (Li et al., 2010; Kim et al., 2018), and in the formation of the nervous system in conjunction with miR-184 (Li et al., 2016, 2019). LncRNA UCA1 promotes cell proliferation by upregulating Foxo3 and downregulating miR-96 (Zhou et al., 2018). Furthermore, miR-96, along with miR-200 (target *kirrel*; *kirrel1*) mutations, causes steroid-resistant nephrotic syndrome (Solanki et al., 2019) and is essential for steroid synthesis during early sex differentiation in tilapia (Tao et al., 2016). *mfap4* is also shown to play an important role in the innate immune system of zebrafish (Zakrzewska et al., 2010; Li et al., 2014; Walton et al., 2015) and catfish (Niu et al., 2011). Foxo3a and Foxo3b in ovarian follicular cells during vitellogenesis were significantly increased stage dependently and co-localized with CYP19a1a (Liu et al., 2016). *foxo3b* retained most of the functions including upregulating *cyp19a1a* during vitellogenesis of orange-spotted grouper (Liu et al., 2016). WB revealed that overexpression of miRNA-96 substantially reduced FOXO3 protein expression (Yin et al., 2020). In the present study, qRT-PCR and miRNA-seq indicated that miRNA-96 decreased, while *foxo3* increased, in ovaries of rare minnow after MT exposure. The miR-183 cluster (miR-96/183)–*foxo3* pair presented in this study revealed that its pathway may result in damage to the central nervous system (Li et al., 2010), immune impairment, or even cancer (Dambal et al., 2015; Ichiyama and Dong, 2019; Zou et al., 2019). The latest study showed that miR-101 regulated *STAR*, *CYP19A1*, *CYP11A1*, and *3*β*-HSD* steroid hormone synthesis-associated genes by *STC1* depletion, thus, promoting E2 secretions (An et al., 2020). These results suggest that MT regulates related gene expression by interfering with miRNAs, thereby, injuring the ovary.

The members of miR-203, miR-204, and miR-205 targeted the *mfap4* gene, which is involved in immune response (Kang et al., 2019) through miR-203–Irak4–Nf-κB-mediated signaling (Xu et al., 2018). Zebrafish miR-203 targeted *dmrt2b* associated with muscle differentiation (Lu et al., 2017), *pax6b* related to retina development (Rajaram et al., 2014), as well as the Wnt signaling transcription factor *lef1* essential for caudal fin regeneration (Thatcher et al., 2008). Moreover, miR-183 cluster, together with miR-203 (target *mfap4*), is expressed in normal T cells involved in C/EBPβ pathway (Steinhilber et al., 2015) through the suppressor of cytokine signaling-3 (Socs-3, Sonkoly et al., 2007). Our results suggest that miR-203 may, thus, be involved in the process of immune response, organic differentiation, and development. *Cyp19a1a* directly participates in the regulation of sexual reproduction in teleost fish (Liu S. et al., 2014; Liu Y. et al., 2014). Estradiol-17β (E2) is produced by conversion of androgen via cytochrome P450 aromatase, encoded by *cyp19a1a*. Thus, the expression of *cyp19a1a* and E2 secretion plays important roles in sex differentiation, gonadal development, and sex reversal. The transcriptional modulation of steroidogenic enzymes in response to MT could be triggered by factors in the HPG axis.

CONCLUSION

In general, in this study, 73,449 unigenes, 24 known mature miRNAs, and 897 novel miRNAs of *G. rarus* were identified by integrated analysis of mRNA- and miRNA-seq. Among them, we successfully identified six miRNA–target pairs, which suggests that they might possibly be involved in cell proliferation and development, signal transduction, metabolic and immune processes, and in the development and functioning of the nervous system. *fabp3 and tgfb1* are target genes regulated by miR-19, while *abca1*, *foxo3*, *tgfb1*, and *zfp36l1* are target genes regulated by miR-96, and *mfap4* and *foxo3* are target genes regulated by miR-183, while *mfap4* is also regulated by miR-203. Such mRNAs and miRNAs, corresponding with ovarian development and innate immune response, were tested by qPCR. The differentially expressed miRNAs (miR-183 cluster and miR-203) with MT administration are provided as the novel regulators in the process of ovarian development and innate immune system in *G. rarus*. Altogether, these results will aid to improve the current understanding of the toxicological effects on fish in response to androgen and will lay a foundation for further studies of EDCs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA730106.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Shanxi Agriculture University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SL designed the experiment and wrote the manuscript. QY and YC conducted the qRT-PCR and histological experiment. QL, WW, and JS contributed to sequencing data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.695699/full#supplementary-material

Supplementary Figure 1 | PCA analysis of RNA and miRNA. The variation between samples was constrained in the PCA analysis [**(a)** 24.8 and **(b)** 43.7% of the overall variance for RNA and miRNA; $P < 0.05$]. In both panels, different colors correspond to samples from different MT concentrations. The percentage of variation explained by each axis refers to the proportion of the total data variance explained by the constrained factor.

Supplementary Figure 2 | Heatmaps of RNA **(a)** and miRNA **(b)** ($n = 3$).

Supplementary Figure 3 | Network heatmap of RNA and miRNA. **(a)** Analyzing the correlation between the two modules and drawing a heat map. Each row and column represents a module, the number in the box is the correlation coefficient of the two modules, and the number in brackets is the *p*-value. The darker the square color is (red or green), the stronger the correlation is; the lighter the square color, the weaker the correlation. The *p*-value of the correlation between the two modules is calculated by student's *t*-test. The smaller the p-value, the higher the similarity between the two modules. **(b)** Heat map drawn from clusters of the module genes. Each row and column represent a gene, and the darker the color of each point (white→yellow→red) represents the stronger connectivity between the two genes corresponding to the row and column. *P*-value was calculated by student's *t*-test. The smaller the *p*-value, the more significant the correlation between the gene and the module. **(c)** The value of module eigenvalue in each sample reflects the comprehensive expression level of all genes in each sample. The abscissa is the sample, the ordinate is the module, and the characteristic value of the module is used for drawing. Red represents high expression and green represents low expression. The graph directly reflects the expression mode of each module in each sample.

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A Modern Genotoxicity Testing Paradigm: Integration of the High-Throughput CometChip® and the TGx-DDI Transcriptomic Biomarker in Human HepaRG™ Cell Cultures

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Higher-throughput, mode-of-action-based assays provide a valuable approach to expedite chemical evaluation for human health risk assessment. In this study, we combined the high-throughput alkaline DNA damage-sensing CometChip® assay with the TGx-DDI transcriptomic biomarker (DDI = DNA damage-inducing) using high-throughput TempO-Seq®, as an integrated genotoxicity testing approach. We used metabolically competent differentiated human HepaRG™ cell cultures to enable the identification of chemicals that require bioactivation to cause genotoxicity. We studied 12 chemicals (nine DDI, three non-DDI) in increasing concentrations to measure and classify chemicals based on their ability to damage DNA. The CometChip® classified 10/12 test chemicals correctly, missing a positive DDI call for aflatoxin B1 and propyl gallate. The poor detection of aflatoxin B1 adducts is consistent with the insensitivity of the standard alkaline comet assay to bulky lesions (a shortcoming that can be overcome by trapping repair intermediates). The TGx-DDI biomarker accurately classified 10/12 agents. TGx-DDI correctly identified aflatoxin B1 as DDI, demonstrating efficacy for combined used of these complementary methodologies. Zidovudine, a known DDI chemical, was misclassified as it inhibits transcription, which prevents measurable changes in gene expression. Eugenol, a non-DDI chemical known to render misleading positive results at high concentrations, was classified as DDI at the highest concentration tested. When combined, the CometChip® assay and the TGx-DDI biomarker were 100% accurate in identifying chemicals that induce DNA damage. Quantitative benchmark concentration (BMC) modeling was applied to evaluate chemical potencies for both assays. The BMCs for the CometChip® assay and the TGx-DDI biomarker were highly concordant (within 4-fold)

and resulted in identical potency rankings. These results demonstrate that these two assays can be integrated for efficient identification and potency ranking of DNA damaging agents in HepaRG™ cell cultures.

Keywords: genetic toxicology, TGx-DDI genomic biomarker, TGx-28.65 genomic biomarker, metabolic activation, toxicogenomics, human health risk assessment

INTRODUCTION

New tools and approaches are urgently needed to allow regulatory agencies worldwide to evaluate a backlog of chemicals for potential adverse human health effects (1–7). Twenty-first century toxicology requires more affordable tests that are higher-throughput, higher-content, human-relevant, and mechanistic in nature for effective chemical evaluation (8–13). Applying *in vitro* toxicogenomic (TGx) biomarkers in metabolically competent human cells in culture is a new approach methodology (NAM) that can help to accomplish these goals. Transcriptomic biomarkers are defined gene sets that produce reproducible changes for altered key events in adverse outcome pathways. These biomarkers can be used to identify chemical mode of action (MoA) and to guide chemical prioritization and classification (14–19). The use of *in vitro* genomic biomarkers to predict specific toxicological responses reduces the subjectivity of interpretation for complex genomic data sets and can thus facilitate the use of genomics for human health risk assessment.

Genetic damage can lead to mutagenicity and genome instability, which in turn can result in adverse human health effects, such as inherited genetic diseases and cancer (20). Consequently, genotoxicity testing is a critical component of chemical evaluation. Genotoxicity and mutagenicity testing depends on jurisdiction, but generally includes the Ames bacterial reverse mutation assay, an *in vitro* mammalian genotoxicity assay (e.g., chromosome aberrations, micronuclei (MN), and/or gene mutations), and an *in vivo* rodent genotoxicity assay [e.g., chromosome aberrations, MN, and/or transgene mutations; (21–24)]. These tests are not typically high-throughput and generally do not provide mechanistic insight into a test compound's MoA. Higher-throughput, mechanism-based genotoxicity tests in human cell culture models can aid in the interpretation of these assays to determine potential human risk. More recent advances to modernize genetic toxicity assays have begun to address this need; for example, the *in vitro* MicroFlow® micronucleus assay (25–28) and the *in vitro* CometChip® assay (29–33) are compatible with various human and rodent cell lines and are relatively higher-throughput methods to assess DNA damage.

A long-term goal is to have a suite of transcriptomic biomarkers that enable rapid extraction of MoA and hazard information from high-throughput transcriptomic (HTTr) screens (7). The TGx-DDI biomarker provides an alternative approach wherein potentially genotoxic MoAs can be discerned using transcriptomic data sets. The TGx-DDI transcriptomic biomarker was developed from a training set of global gene expression profiles from human TK6 cells exposed to 28 prototype DNA damage-inducing (DDI) or non-DDI chemicals (34–37). The biomarker comprises 64 genes; changes in the expression of these genes can be used to classify chemicals as DDI or non-DDI using a variety of gene expression technologies in TK6 cells, including DNA microarrays (35), quantitative PCR arrays (38) and the high-throughput NanoString nCounter® platform (36). Overall, it has been proposed that the TGx-DDI biomarker can be used in a variety of contexts including chemical screening (39), hazard identification, chemical prioritization for further testing, MoA development, weight of evidence analysis, and/or potency assessment (36).

Currently, tremendous efforts are being made to develop suitable cell-based assays as a reliable and informative substitute for *in vivo* studies. Although, human TK6 cells are a suitable choice of cell line to evaluate genotoxicity for regulatory applications, a substantial limitation is that they lack metabolic activity. Primary human hepatocytes, often considered the gold standard for physiologically-relevant *in vitro* liver cell culture models, also have some notable limitations in that they have a finite supply from an individual donor making them difficult to obtain in large quantities for year-over-year evaluations, have a highly limited lifespan of differentiated functionality in conventional 2D culture models (∼3–5 days), and in some countries are not an ethically viable option (40–42). A suitable alternative is to use human HepaRG™ cells, which were derived from a hepatocellular carcinoma in a Caucasian female donor (43). Under differentiating conditions, HepaRG™ cell cultures express relevant amounts of Phase I and Phase II metabolic enzymes, transporters and nuclear receptors, and differentiate into co-cultures of hepatocyte- and cholangiocyte-like cells, which makes them a suitable choice for toxicity screening (43). HepaRG™ cells retain many characteristics of primary human hepatocytes and thus circumvent the need to add rat liver S9, which can be problematic for some compounds and is a limitation of the TK6 cell line. Moreover, HepaRG™ cells have undergone extensive validation for *in vitro* cytochrome P450 induction and have been deemed a reliable human cell line in terms of metabolic competence (44–51). This was further confirmed by an interlaboratory validation of liver enzyme induction models led by the European Commission, Joint Research Center (52). The popularity of these cells in toxicology studies is thus growing, and they are currently being used in HTTr screens. For example, Ramaiahgari et al. (53) used high-throughput targeted RNA-sequencing (TempO-Seq®;

Abbreviations: AFB1, Aflatoxin B1; BaP, Benzo[a]pyrene; CISP, Cisplatin; CP, Cyclophosphamide; AraC, Cytosine Arabinoside; DDI, DNA damage-inducing; 2DG, 2-Deoxy-D-Glucose; HC, hierarchical clustering; MMS, Methyl methanesulfonate; ENU, N-Nitroso-N-Ethylurea; EUG, Eugenol; PG, Propyl Gallate; ZDV, Zidovudine; HTTr, High-Throughput Transcriptomics; MN, Micronuclei; MoA, Mode of Action; NAM, New Approach Methodology; non-DDI; non-DNA damage-inducing; NSC, Nearest Shrunken Centroids; PCA, Principal Component Analysis; SSBs, Single Strand Breaks; TGx, toxicogenomics.

TABLE 1 | Test chemical information for Group 1 (genotoxic/DDI chemicals), Group 2 (non-genotoxic/non-DDI chemicals), and Group 3 (misleading/irrelevant positive chemicals) based on the recommended genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests by Kirkland et al. (57, 58).

	Chemical use/formation	Chemical effects	Kirkland et al. (57, 58) chemical group
Aflatoxin B1	Food contaminant produced by pathogenic fungus	Forms DNA adducts; clastogenic, mutagenic, teratogenic, carcinogenic	Group 1
Benzo[a]pyrene	Polycyclic aromatic hydrocarbon; formed during incomplete combustion	Forms DNA adducts; clastogenic, mutagenic, carcinogenic	Group 1
Cisplatin	Chemotherapeutic agent	Alkylating agent that interferes with DNA replication; cross-linking agent; clastogenic and mutagenic	Group 1
Cyclophosphamide	Chemotherapeutic agent	Alkylating agent; clastogenic, mutagenic	Group 1
Cytosine arabinoside	Chemotherapeutic agent	DNA anti-metabolite that interferes with DNA replication; clastogenic	Group 1
Methyl methanesulfonate	Chemotherapeutic agent	Alkylating agent; clastogenic, mutagenic, carcinogenic	Group 1
N-Ethyl-N-nitrosourea	Chemotherapeutic agent	Alkylating agent; clastogenic, mutagenic, carcinogenic and teratogenic	Group 1
Zidovudine (Azidothymidine)	Anti-HIV drug	Nucleoside reverse transcriptase inhibitor (NRTI); clastogenic	Group 1
Propyl gallate	Antioxidant; food additive	Used to prevent oxidation; mutagenic and clastogenic	(Group 1)[#]
2-Deoxy-D-glucose	Used as a diagnostic agent in its radiolabelled form	Investigational drug that is being studied as an anticancer and antiviral agent; glycolysis inhibitor	Group 2*
Eugenol	Naturally occurring phenolic molecule found in plants; local analgesic agent to alleviate tooth pain	Interferes with action potential conduction; has anti-inflammatory, neuroprotective, antipyretic, antioxidant, antifungal and analgesic properties	Group 3
Urea	Organic compound important in the metabolism of nitrogen-containing compounds by animals	Nitrogen-containing substance in mammalian urine, also used in fertilizers; non-toxic	Group 3

[#]*PG was removed from Group 3 in the Kirkland et al. (58) updated recommended lists, as PG is now reported to be positive in the Ames test in the presence of S9 and induces micronuclei and chromosomal aberrations in vivo. We have thus included PG as Group 1.*

**Chemical not included in Kirkland et al. (57, 58) recommended lists for non-genotoxic chemical, but 2-deoxy-D-glucose fits the criteria to be included in Group 2 (non-genotoxic chemical) and is used as a non-DDI reference chemical in the development of the TGx-DDI biomarker in Li et al. (35).*

Templated Oligo-Sequencing) in HepaRG™ cells to conduct concentration-response modeling for 24 reference compounds to explore transcriptomic characteristics distinguishing compounds that result in drug-induced liver injury.

The use of the TGx-DDI genomic biomarker in metabolically competent human HepaRG™ cell culture is advantageous. Our pilot work showed that the biomarker was 100% accurate in identifying five DDI and five non-DDI toxicants in HepaRG™ cells by RNA-sequencing (54). Corton et al. (39) also demonstrated predictive accuracies of 90% in identifying DDI agents in HepaRG™ cells using the TGx-DDI biomarker in combination with a pattern matching correlation approach. Nevertheless, additional validation studies that confirm the accuracy of the TGx-DDI biomarker in human HepaRG™ cells using the most recent HTTr platforms would be tremendously useful to advance its application in such chemical screens for genotoxic hazard identification.

The comet assay offers an alternative approach to genotoxicity testing that directly tests for the presence of physical damage to DNA. We reasoned that together, these two approaches would be highly complementary, providing an efficient integrated test to accurately identify genotoxic agents. While the traditional comet assay is not compatible with high-throughput screens due to the need for a single glass slide for each condition, the recently available high-throughput CometChip® platform has >200x the capacity of the traditional comet assay for identifying chemicals that induce DNA strand breaks and has been extensively validated (30–33, 55, 56). The objectives of the present study are thus to: (1) extend validation efforts of the TGx-DDI genomic biomarker further through analysis of HepaRG™ cells exposed to prototype DDI and non-DDI agents; (2) confirm the predictive accuracy of TGx-DDI using the TempO-Seq® platform; (3) explore the integration of the high-throughput alkaline CometChip® assay and the TGx-DDI biomarker as an efficient, next-generation genotoxicity screening approach to identify DDI chemicals; and (4) conduct concentration-response modeling to investigate chemical potency ranking for DNA damage measured by the CometChip® assay vs. transcriptional changes in the TGx-DDI biomarker genes.

Herein we investigate 12 test chemicals with varied MoAs, chemical uses, and effects (**Table 1**). For practical purposes, we classified the test chemicals as either DDI or non-DDI. All DDI test compounds are Group 1 chemicals based on the Kirkland et al. (57, 58) recommended lists of genotoxic chemicals for the assessment of the performance of new or improved genotoxicity tests and should render a positive result in mammalian genotoxicity tests in culture. In this study, the DDI chemicals include: aflatoxin B1 (AFB1), benzo[a]pyrene

(BaP), cisplatin (CISP), cyclophosphamide (CP), cytosine arabinoside (AraC), methyl methanesulfonate (MMS), N-nitroso-N-ethylurea (ENU), and zidovudine (ZDV; also known as azidothymidine). The non-DDI group of chemicals in this study can be further broken down into one well-established non-DDI chemical (i.e., Group 2) and three potentially misleading (irrelevant) positives (i.e., Group 3) based on the Kirkland et al. (57, 58) recommended list of non-genotoxic chemicals. In this study, 2-deoxy-D-glucose (2DG) is a non-DDI Group 2 chemical based on the criteria presented in the Kirkland et al. (57, 58) reports, as 2DG is expected to render a negative result in *in vitro* human and rodent cell-based genotoxicity tests. Group 3 chemicals should also yield negative results in genotoxicity tests with mammalian cells. Chemicals in Group 3 are most often negative in the Ames assay and *in vivo*; however, chemicals in this grouping have been reported to induce DNA damage, most often at high concentrations or with high levels of cytotoxicity, which leads to "misleading" positive results. Based on the Kirkland et al. (57) recommended lists, eugenol (EUG), propyl gallate (PG), and urea were all considered to be Group 3 chemicals that have the potential to result in misleading positive results. However, based on the updated recommended chemical list published by Kirkland et al. (58), PG is reported as positive in the Ames test with S9 and induces MN and chromosomal aberrations *in vivo* (59, 60). Thus, PG has now been removed from Group 3, as it is potentially DNA-reactive and positive *in vivo* for certain genotoxic endpoints. Although, PG is not a Group 1 reference chemical, we expect it to classify as a Group 1; hence, we have grouped PG with the DDI chemicals herein.

Taken together, we present results for nine DDI, one non-DDI and two potentially misleading DDI agents. We found that combining a DNA damage assay that rapidly detects DNA strand breaks (i.e., the CometChip®) with the TGx-DDI genomic biomarker in metabolically competent HepaRG™ cells provides and efficient and accurate approach to identify and rank potencies of chemicals.

MATERIALS AND METHODS

Chemicals

Test chemicals were purchased from Cayman Chemical (CISP; Ann Arbor, MI, USA), TCI America (PG; Montgomeryville, PA, USA), and Millipore Sigma (remaining chemicals; St. Louis, MO, USA) for exposures in fully differentiated, cryopreserved No-Spin HepaRG™ cells (Triangle Research Labs (TRL), Durham, NC, USA; acquired by Lonza Bioscience). Test chemical information, including corresponding vehicle control and concentrations tested are shown in **Table 2**. The chemical exposures in HepaRG™ cells, the cell viability studies, and the paired high-throughput CometChip® analysis were conducted at Integrated Laboratory Systems, Inc. (ILS; Research Triangle Park, Durham, NC, USA).

HepaRG™ Cell Culture and Chemical Exposures

Human HepaRG™ cell cultures were exposed to increasing concentrations of 12 test chemicals in parallel 96-well plates (four test chemicals per plate) for assessment of DNA damage by CometChip® and for collection of cell lysates for TempO-Seq® analysis for TGx-DDI classification purposes. Concentration setting for each test chemical was based on data previously collected at ILS and was established from the observation of either a robust positive CometChip® response or an upper concentration that was approaching (but not above) an overt cytotoxicity threshold (<40% viable cells) in previous in-house studies. In the absence of a positive CometChip® response or cytotoxicity, chemicals were tested up to a top concentration of 10 mM, which is compliant for non-cytotoxic, negative compounds in OECD test guidelines for mammalian cell assays (61, 62). Briefly, differentiated human HepaRG™ cells, derived from a hepatocellular carcinoma (45) were thawed and seeded at ∼4.0–5.0 × 10^4 viable cells per well in a collagen-coated 96-well CometChip® in William's E medium with TRL's Thawing and Plating Supplement. Cells were incubated for 7 days following seeding to allow the cells to regain peak metabolic function (45). Cells were then exposed in culture medium containing TRL's Pre-Induction/Tox Supplement to five concentrations of each DDI or non-DDI chemical daily in a repeated exposure design (exposures at 0, 24, and 48 h). Four hours following the last treatment (52 h total time), one plate of cells was used for cell viability ($n = 2$ per treatment group alongside matched solvent controls) and CometChip® analysis and the second plate was used to generate cell lysates for gene expression analysis ($n = 4$ per treatment group per assay for CometChip® and TempO-Seq®). The media was aspirated from exposed cells and they were washed with PBS, prior to adding 100 µl of TrypLE™ (for cell viability and CometChip® assays; ThermoFisher Scientific, Waltham, MA, USA) or 1X TempO-Seq® Lysis Buffer in PBS (for TempO-Seq® assay; BioSpyder Technologies, Carlsbad, CA, USA) to each well to lyse cells for 10 min at room temperature. Cell lysates were then frozen and stored at −80°C for subsequent transcriptome profiling described below. Samples used for the analysis of cell viability and DNA damage using CometChip® were neutralized with the addition of 100 µl of culture medium to each well and were processed as described in the following sections.

Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to determine the number of viable HepaRG™ cells based on the quantification of ATP present following each chemical treatment. Cytotoxicity was evaluated 4 h after the last exposure following the manufacturer's instructions in 96-well plates. Briefly, wells containing 100 µl cell samples were equilibrated at room temperature for 30 min prior to the addition of CellTiter-Glo® Reagent to each well in a volume equal to that of the cell culture medium (e.g., 100 µl). The contents were mixed for 2 min on an orbital shaker to induce cell lysis prior to incubation at room temperature for 10 min to stabilize the luminescent signal. Luminescence was measured on a SpectraMax® plate reader (Molecular Devices, San Jose, CA, USA). Luminescent signal is the result of the release of ATP from metabolically active cells and is directly proportional to the

TABLE 2 | Experimental information for DDI (genotoxic) and non-DDI (non-genotoxic) test chemicals used in this study.

Test chemical	Chemical abbreviation	CAS No.	Chemical group#	Vehicle control	Concentrations tested (μm)
Aflatoxin B1	AFB1	1162-65-8	Group 1	DMSO	3.125, 6.25, 12.5, 15, 25
Benzo[a]pyrene	BaP	50-32-8	Group 1	DMSO	0.9375, 1.875, 3.75, 7.5, 15
Cisplatin	CISP	15663-27-1	Group 1	DMSO	3.125, 6.25, 12.5, 25, 50
Cyclophosphamide	CP	6055-19-2	Group 1	DMSO	1,250, 2,500, 5,000, 7,500, 10,000
Cytosine arabinoside	AraC	147-94-4	Group 1	DMSO	12.5, 25, 50, 100, 200
Methyl methanesulfonate	MMS	66-27-3	Group 1	DMSO	22.7, 45.4, 90.8, 181.6, 363.2
N-Nitroso-N-ethylurea	ENU	759-73-9	Group 1	DMSO	312.5, 625, 1,250, 2,500, 5,000*
Zidovudine (azidothymidine)	ZDV	30516-87-1	Group 1	DMSO	125, 250, 500, 1,000, 2,000
Propyl gallate	PG	121-79-9	(Group 1)	DMSO	125, 250, 500, 750, 1,000*
2-Deoxy-D-glucose	2DG	154-17-6	Group 2	Water	625, 1,250, 2,500, 5,000, 10,000
Eugenol	EUG	97-53-0	Group 3	DMSO	156.25, 312.5, 625, 1,250, 2,500*
Urea	Urea	57-13-6	Group 3	DMSO	625, 1,250, 2,500, 5,000, 10,000

#*Chemical grouping based on Kirkland et al. (57, 58) recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests.*

**Indicates a cytotoxic concentration (<40% cell viability; >60% cytotoxic) that was subsequently eliminated from the gene expression analysis; all concentrations were used for CometChip® analysis.*

number of viable cells in the culture. The cytotoxicity cut-off was >60% cytotoxic (equivalent to <40% viable cells).

Trevigen CometChip® Assay

Exposed and control HepaRG™ cells were loaded into the CometChip® wells and were allowed to settle into microwells of a 96-well CometChip®. A 1% agarose overlay was then applied and the cells were lysed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 (Sigma, St. Louis, MO, USA) and 10% DMSO) overnight at 4°C. Following lysis, the CometChip® was equilibrated in an alkaline electrophoresis buffer (300 mM NaOH/1 mM EDTA) for 40 min and electrophoresed for 50 min under a 300 mA current at 4°C. These alkaline conditions are used to detect DNA single strand breaks (SSBs). Following electrophoresis, the CometChip® was neutralized at 4°C for 2 × 15 min in 0.4 M Tris, pH 7.4 and equilibrated overnight at 4°C in 20 mM Tris, pH 7.4. Once equilibrated, the chip was stained for 30 min at 4°C in 0.1X SYBR Gold and then destained for >1 h at 4°C in 20 mM Tris, pH 7.4. After destaining, images were taken at 4X magnification of all 96 wells. The tiff images were captured and analyzed using Trevigen® Comet Analysis Software.

Statistical Analysis of CometChip® Data

The median % tail DNA for the CometChip® data was analyzed using one-way analysis of variance (ANOVA). The Anderson-Darling statistic was used to test the normality assumption and the Fligner-Killeen test of homogeneity of variances was used to test the common variance assumption. If either assumption was not satisfied, the rank transformation was applied and the non-parametric one-way ANOVA was performed (63). All pairwise comparisons to matched vehicle controls were conducted using the *t*-test. The resulting *p*-values were then FWER adjusted using the Dunnett's method.

TempO-Seq® Library Preparation and S1500+ Targeted Transcriptome Sequencing

The TempO-Seq® Human Tox+Surrogate Panel Reagent Kit (BioSpyder Technologies, Carlsbad, CA, USA) was used to prepare libraries in a 96-well plate format from exposed and control HepaRG™ cell lysates, according to the manufacturer's instructions. All five concentrations of each test chemical were used for gene expression analysis, except for overtly cytotoxic concentrations that were eliminated from the analysis [i.e., the highest concentration (C5) of ENU, EUG and PG; **Table 2**]. Assay controls included a negative no-lysate control (1X TempO-Seq® Lysis Buffer only), and two positive controls: qPCR Human Reference Total RNA and Human Brain Total RNA (Takara Bio, CA, USA; four replicates per control). Briefly, 2 μl of cell lysate in 1X TempO-Seq® Lysis Buffer from each treatment and concentration were hybridized with the targeted Human S1500+ Tox Panel detector oligo (DO) probe mix (v1.1; 2,977 probes), for 10 min at 70°C followed by a temperature gradient with a ramp rate of 0.5°C/min to 45°C over 50 min followed by a nuclease digestion to remove excess, unbound, or incorrectly bound DOs enzymatically at 37°C for 90 min. The DO pairs bound to adjacent target sequences were then ligated (60 min at 37°C, followed by a 15 min enzyme denaturation at 80°C) to generate a pool of amplification templates. Each amplification template (10 μl of ligated DOs) was transferred to its respective well of the 96-well PCR plate containing PCR Pre-Mix and Primers. Amplification was conducted using a CFX96 Real-Time PCR Detection System (Bio-Rad, Mississauga, ON, Canada) to add a sequence tag unique to each sample and the sequencing adaptors using the following PCR program settings: 37°C for 10 min, 95°C for 2 min; 6 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 120 sec; 16 cycles of 95°C for 30 sec; 72°C for 2 min; 72°C for 1 min. All 288 TempO-Seq® libraries prepared from the three 96-well plates were pooled (5 μl of each sample)

and purified using the Macherey-Nagel NucleoSpin® Gel and PCR Clean-Up kit (Clontech Laboratories Inc., Bethlehem, PA, USA), according to the manufacturer's directions for PCR clean-up with three modifications outlined in the TempO-Seq® Assay User Guide. The pooled, purified TempO-Seq® libraries were sequenced on two NextSeq® 500/550 High Output flow cells (v2 kits, 75 cycles) using an Illumina NextSeq® 500 Sequencing platform (Illumina, San Diego, CA, USA).

Sequencing Data Preprocessing, Alignment, and Quality Control

Sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database under accession number GSE171360. Raw sequencing data were demultiplexed (i.e., assigned to respective sample files) with blc2fastq v2.20.0.422, and trimmed for quality control using fastp (v0.20.0). The resulting FASTQ files were aligned to reference sequences for the TempO-Seq® Human Tox+Surrogate Panel (2,977 probes) provided by BioSpyder using their purpose-built analysis pipeline (TempO-SeqR, v3.0) to generate a table of counts per gene per sample. Briefly, this pipeline used STAR v2.7.8a to perform alignment of raw reads to the reference sequences, and the qCount function of the QuasR package (v1.30.0) to produce a gene X sample count matrix of raw counts from the BAM files output by STAR.

Study-wide quality control was performed on the count matrix using several methods to measure consistency and remove low-quality samples, using Harrill et al. (64) as a guideline. Samples that clustered as singletons at a dissimilarity of 0.1 using 1-Spearman correlation using complete linkage were removed from the study. As described by Harrill et al. (64), we used a cutoff of uniquely mapped reads as 10% of the number of target sequences (i.e., 100,000 reads to pass filter, because the target is 1,000,000 for TempO-Seq® experiments). We removed any samples outside of Tukey's Outer Fence (3X interquartile range) for: (1) the number of probes capturing the top 80% of the signal; (2) the Gini coefficient (which measures inequality in distributions); and (3) the number of active probes (those with at least 5 mapped reads). Based on these metrics, a single experimental sample was removed (one replicate of EUG C4).

The code used to perform processing of high-throughput sequencing data is available at https://github.com/mattjmeier/2021_Buick_et_al_HepaRG_CometChip_TGx-DDI.

Statistical and Bioinformatic Analyses for TGx-DDI Classification

Read counts were normalized using DESeq2 (v1.30.1) (65) using the counts() function in R (66) to account for sequence-to-sequence variability in read depth between the samples. Samples with sub-optimal sequencing depth (total number of reads $<$ 500 K) or that were overtly cytotoxic ($>$60% cytotoxic; $<$40% viable cells) were excluded from the analysis. Data visualization using boxplots and hierarchical cluster analysis were conducted to identify samples with poor data quality. This resulted in the exclusion of one sample (a replicate of EUG C4) from the TGx-DDI classification analysis due to sub-optimal sequencing depth. One replicate of CISP C2 was also excluded from the analysis, as it was identified as "a point of high leverage" outlier (**Supplementary Figure 1**). Statistical modeling and bioinformatics tools were used to classify chemicals as DDI or non-DDI using the TGx-DDI genomic biomarker. Detailed information about the analyses can be found in Yauk et al. (67) and Buick et al. (68). Gene Symbols that had multiple probes for TGx-DDI biomarker genes were averaged. Hierarchical clustering was completed using the hclust() function in R (www.r-project.org). Agglomerative clustering was based on average linkage with Euclidean distances (69). Classifications (DDI vs. non-DDI) were achieved using the Nearest Shrunken Centroids (NSC) method (70) in the pamr function of R (www.bioconductor.org), as has been described previously (36, 67, 68). Briefly, the standardized centroid (SC) was calculated by applying the NSC method for DDI and non-DDI test chemicals in the training set and is the mean expression level for each gene in a class divided by its within-class standard deviation. For each DDI and non-DDI test article, the SC is shrunken in the direction of the overall centroid to create the NSC. Treated and control samples were then classified by comparing their gene expression profile to the class of NSCs and then assigned to a class closest to it in squared distance so that the probability of class membership was $>$0.90 (35, 36).

Three separate analyses were conducted to classify the compounds using the TGx-DDI biomarker, including NSC probability analysis (PA; visualized using heatmaps), principal component analysis (PCA), and hierarchical clustering (HC), as outlined in Yauk et al. (67) and Buick et al. (68). PCA was completed using the prcomp() function in R (71), where the training set data (35) was used to estimate the principal components (PC). These PC loadings were applied to the data generated with the 12 test compounds. A scatterplot generated using data from the TGx-DDI training set and test chemicals was generated to visualize the results. Classification was completed as follows: if a chemical resulted in a positive call in any one of three classification analyses (NSC PA heatmaps, PCA, or HC), it was classified as DDI; whereas, a chemical was classified as non-DDI if it did not lead to a positive result in any of the aforementioned analyses (54, 67, 68).

Benchmark Concentration Modeling of CometChip® Data

Benchmark concentration analysis of CometChip® data (BMC_{CC}) was conducted using BMDExpress v2.3 (https://github.com/auerbachs/BMDExpress-2/releases) following BMD technical guidance (72, 73). Test chemicals with statistically significant increases in median % tail DNA were included for BMC_{CC} modeling with the exception of overtly cytotoxic concentrations, which resulted in the highest concentration (C5) of ENU being excluded from the BMC_{CC} analysis (all concentrations of EUG and PG were excluded from this analysis due to a lack of positive response). Concentration-response data were fit to a model that best described the data using the following models: Linear, Exponential (2, 4, and 5), 2°

Polynomial, and the restricted Power (power restricted to ≥1). The benchmark response (BMR) was set to one standard deviation (1SD) (74). The $BMCU_{CC}$ and $BMCL_{CC}$ values signify the upper and lower 95% confidence limits of the BMC_{CC}, respectively. The "width" of the confidence interval is the distance between the $BMCU_{CC}$ and $BMCL_{CC}$, and therefore defines the BMC_{CC} estimate's precision.

Benchmark Concentration Modeling of TGx-DDI Biomarker Genes

For the TGx-DDI biomarker genes, normalized read counts were shifted by 0.5 and then log2 transformed using the counts() function in the DESeq2 package (65). BMC analysis of TGx-DDI biomarker genes (BMC_{TGx}) was also conducted using BMDExpress v2.3, in accordance with recommendations outlined in the US National Toxicology Program (NTP) Research Report on National Toxicology Program Approach to Genomic Dose-Response Modeling (72, 73). Test chemicals with positive TGx-DDI classifications were included for BMC_{TGx} modeling with cytotoxic concentrations eliminated from the analysis [i.e., the highest concentration (C5) of ENU, EUG, and PG were excluded]. Biomarker genes were analyzed and filtered using the Williams trend test retaining features with a permutation p-value <0.05 (with 250 permutations) with fold changes >1.5. To derive BMC_{TGx} values, TGx-DDI biomarker genes that passed the pre-filters were fit to the following models: Linear, Exponential (2, 4, and 5), 2° Polynomial, and the restricted Power (power restricted to ≥1). A best fit model was selected with the lowest Akaike Information Criterion (AIC) value (lowest complexity). To be consistent with the BMC_{CC} analysis, the BMR was set to 1SD for BMC_{TGx} analysis (74). BMCs were filtered based on the goodness of fit (p-value >0.1), a BMC/BMCL ratio < 20, a BMCU/BMCL ratio < 40, the BMC < the highest concentration, and the BMC could not be < two orders of magnitude lower than the lowest concentration to avoid model extrapolation. A secondary analysis was also conducted in order to generate confidence intervals for the BMC_{TGx} values using the bootstrap method. For each gene, 100 bootstrap samples were generated assuming a normal distribution for each concentration group, where the mean and standard deviation were based on the sample estimates. These data were then imported into BMDExpress v2.3 with the same filtering criteria and model selection as in the BMC_{TGx} analysis. As bootstrap samples are independent, the BMDExpress results were then bootstrapped 2,000 times, where each gene in the biomarker has a probability for inclusion into a bootstrap sample based on the relative frequency of that gene estimated as the total number of BMCs for that gene that passed all the filtering criteria divided by 100. For each bootstrap sample, the median was estimated. From the resulting bootstrap distribution, 95% percentile confidence intervals were obtained for the median BMC_{TGx} (bootstrap).

RESULTS

Human HepaRG™ cells were exposed to increasing concentrations of 12 well-characterized compounds. Exposed cells were analyzed using CometChip® to assess DNA damage and TempO-Seq® for TGx-DDI classification purposes. BMC analyses were conducted for both tests to compare the potency for each test chemical for the different assays.

Identification of Relevant Concentration Ranges for Genotoxicity Testing

Prior to assessing DNA damage and DNA damage-induced genes, it is first necessary to identify concentration ranges that include two or more non-cytotoxic concentrations. Cell viability was assessed using the CellTiter-Glo® Luminescent assay following a 3-day repeat exposure to five concentrations of each test chemical by quantifying the luminescent signal from ATP, an indicator of metabolically active cells, in the treated HepaRG™ cells (**Figure 1**, **Supplementary Table 1**). All of the DDI (Group 1) compounds except ZDV and CISP caused declines in viability (**Figure 1A**). AFB1, BaP, CP, AraC, and MMS resulted in reduced cell viability, but none of the test concentrations exceeded the cytotoxicity threshold of >60% (equivalent to <40% cell viability). The highest concentration (C5) of ENU and PG resulted in <20% cell viability following treatment (**Figure 1**). 2DG (Group 2) and Urea (Group 3) did not cause any notable cytotoxicity at any of the concentrations tested. Finally, EUG (Group 3) caused declines in viability with the top concentration being eliminated due to overt cytotoxicity (<20% cell viability). All concentrations of test chemicals were used for CometChip® analysis, including those causing overt cytotoxicity, for simplicity; however, any chemical treatment exceeding the cytotoxicity limits were excluded from the TempO-Seq® gene expression analysis (i.e., C5 for ENU, EUG, and PG) due to the higher cost of this assay. Cytotoxic concentrations (< 40% viability) were also eliminated for hazard calling and BMC modeling of both endpoints (i.e., C5 for ENU, PG, and EUG were not included; only positive CometChip® and TGx-DDI responses were modeled).

High-Throughput DNA Damage Quantification

Levels of DNA SSBs were quantified in human HepaRG™ cells following repeat exposures to the 12 test chemicals using the alkaline CometChip® assay (**Figure 2**). Chemicals were considered positive if there was an increase in median % tail DNA that was statistically significant compared to matched vehicle control ($p < 0.05$). DNA damage, measured by median % tail DNA, was observed for seven of the nine DDI (Group 1) chemicals to varying degrees, with at least one concentration resulting in significant DNA damage compared to matched vehicle controls. MMS, ENU, and ZDV exposure caused the greatest accumulation of significant DNA damage in HepaRG™ cells ($p < 0.001$; **Figures 2F–H**, respectively; note the y-axis scale for these three compounds is greater than the other chemicals in this figure); however, the top concentration of ENU (C5) surpassed the cytotoxicity threshold (< 20% cell viability). Almost all of the remaining DDI chemicals also induced significant increases in SSBs as detected by CometChip®, but

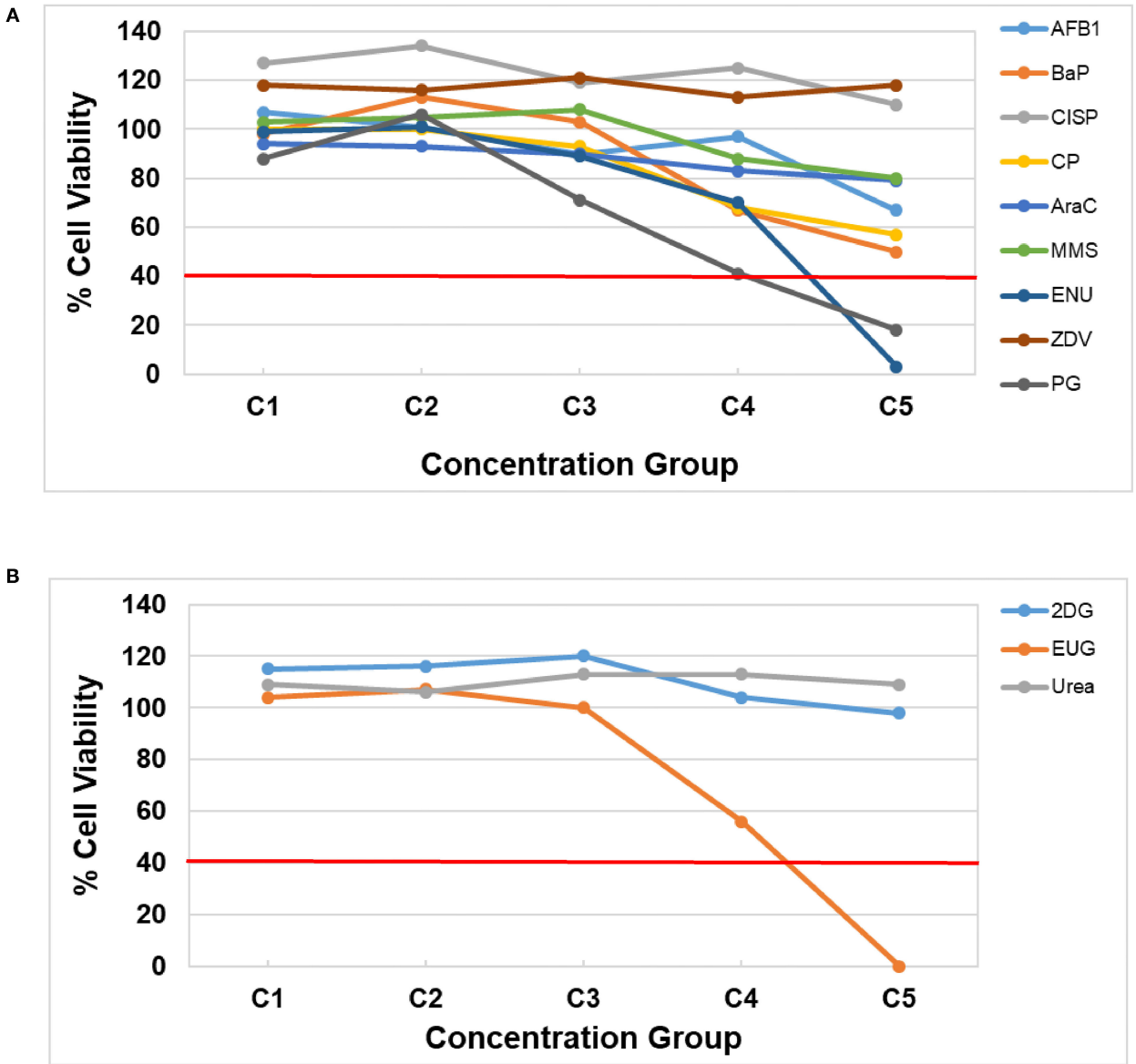

FIGURE 1 | HepaRG™ cell viability measured using the CellTiter-Glo® Luminescent Cell Viability Assay for **(A)** DDI (Group 1) chemicals and **(B)** non-DDI (Group 2 and 3) chemicals. DDI chemical abbreviations: aflatoxin B1 (AFB1), benzo[a]pyrene (BaP), cisplatin (CISP), cyclophosphamide (CP), cytosine arabinoside (AraC), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU), zidovudine (ZVD), and propyl gallate (PG; included with Group 1 chemicals). Non-DDI chemical abbreviations: 2-deoxy-D-glucose (2DG), eugenol (EUG), and Urea. The test concentrations for each chemical are shown in **Table 2** (C1 is the lowest concentration and C5 is the highest concentration). The red line represents the cytotoxicity threshold of <40% cell viability.

to a lesser extent. AraC caused significant DNA damage at the top four concentrations tested (**Figure 2E**). BaP and CISP caused significant increases in % tail DNA at C4 and C5 (**Figures 2B,C**). CP exposure only caused significant % tail DNA increases at the highest concentration ($p < 0.05$; **Figure 2D**). AFB1 exposure did not cause measurable increases in DNA SSBs using the alkaline CometChip® assay (**Figure 2A**), which is consistent with the relatively low magnitude of % tail DNA for agents that induce bulky lesions [this includes AFB1, BaP, CISP, and CP; (55, 75, 76)]. PG exposure also did not yield a statistically significant increase in DNA damage, which is consistent with the fact that it does not directly interact with DNA to create physical damage [**Figure 2I**; (77, 78)].

There was no accumulation of SSBs observed at any of the five concentrations tested for 2DG, the non-DDI chemical (Group 2: non-genotoxic), nor for EUG and Urea (Group 3: misleading positives); median % tail DNA was not statistically increased compared to their matched vehicle controls (**Figures 2J–L**).

In summary, significant DNA damage was detected following exposure to seven out of nine DDI compounds (Group 1). Accumulation of DNA damage was concentration-dependent for the majority of DDI compounds, with very large increases in

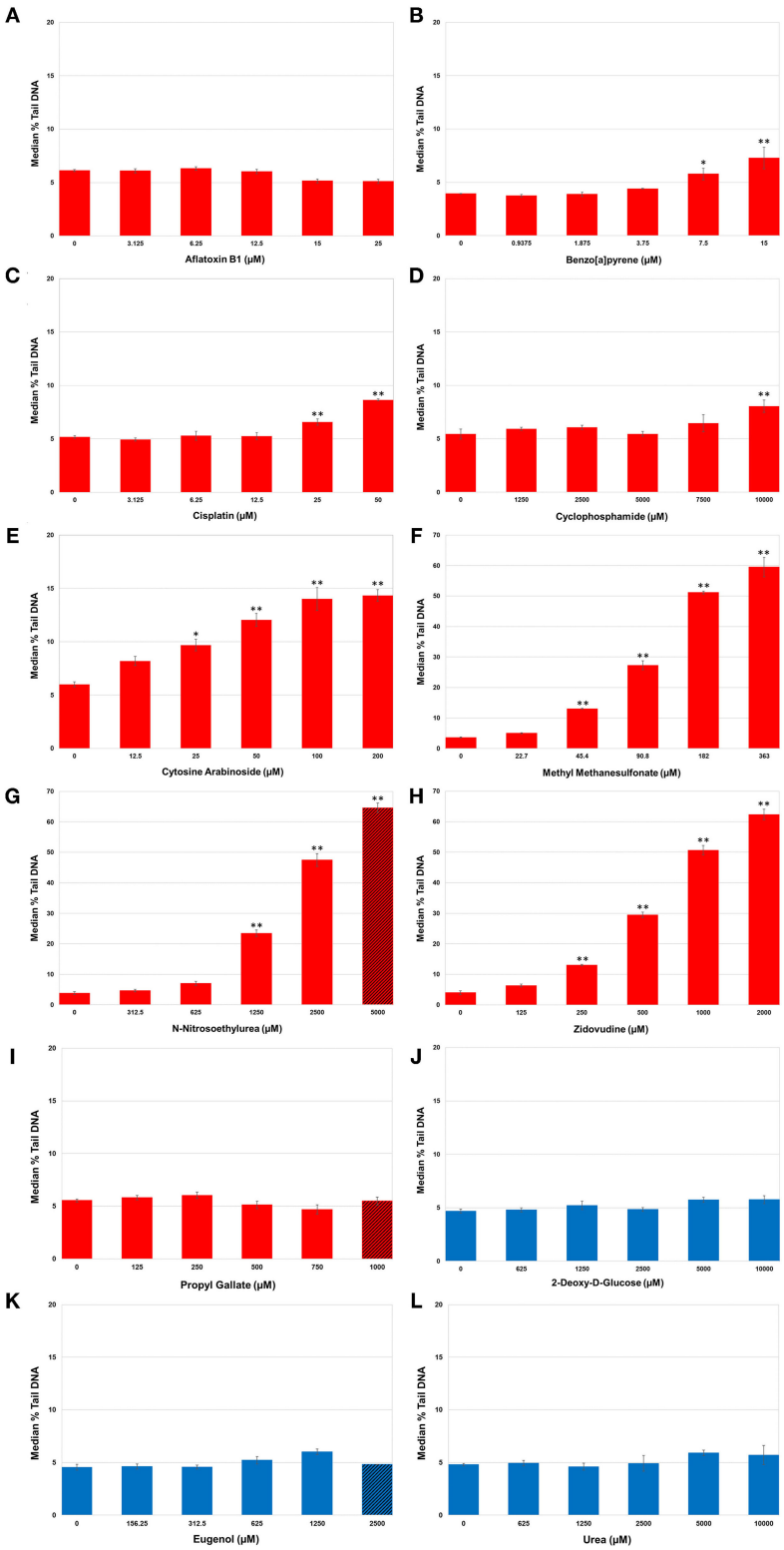

FIGURE 2 | DNA damage in human HepaRG™ cells measured using the alkaline CometChip® assay. Cells were exposed to increasing concentrations of DNA damage-inducing **(DDI; A–I)** and non-DDI test chemicals **(J–L)**. Median % tail DNA is shown 4 h following the last exposure. The data are expressed as the median % tail DNA ± SE ($n = 4$). Red bars represent Group 1 DDI chemicals, blue bars denote the Group 2 non-DDI chemical and the Group 3 potentially misleading positive chemicals. Diagonal lines indicate overtly cytotoxic concentrations. Note the difference in the scale of the y-axis for MMS, ENU, and ZDV due to the large magnitude of the response for these three DDI (Group 1) chemicals. *$P < 0.05$, **$P < 0.001$ compared to matched vehicle control.

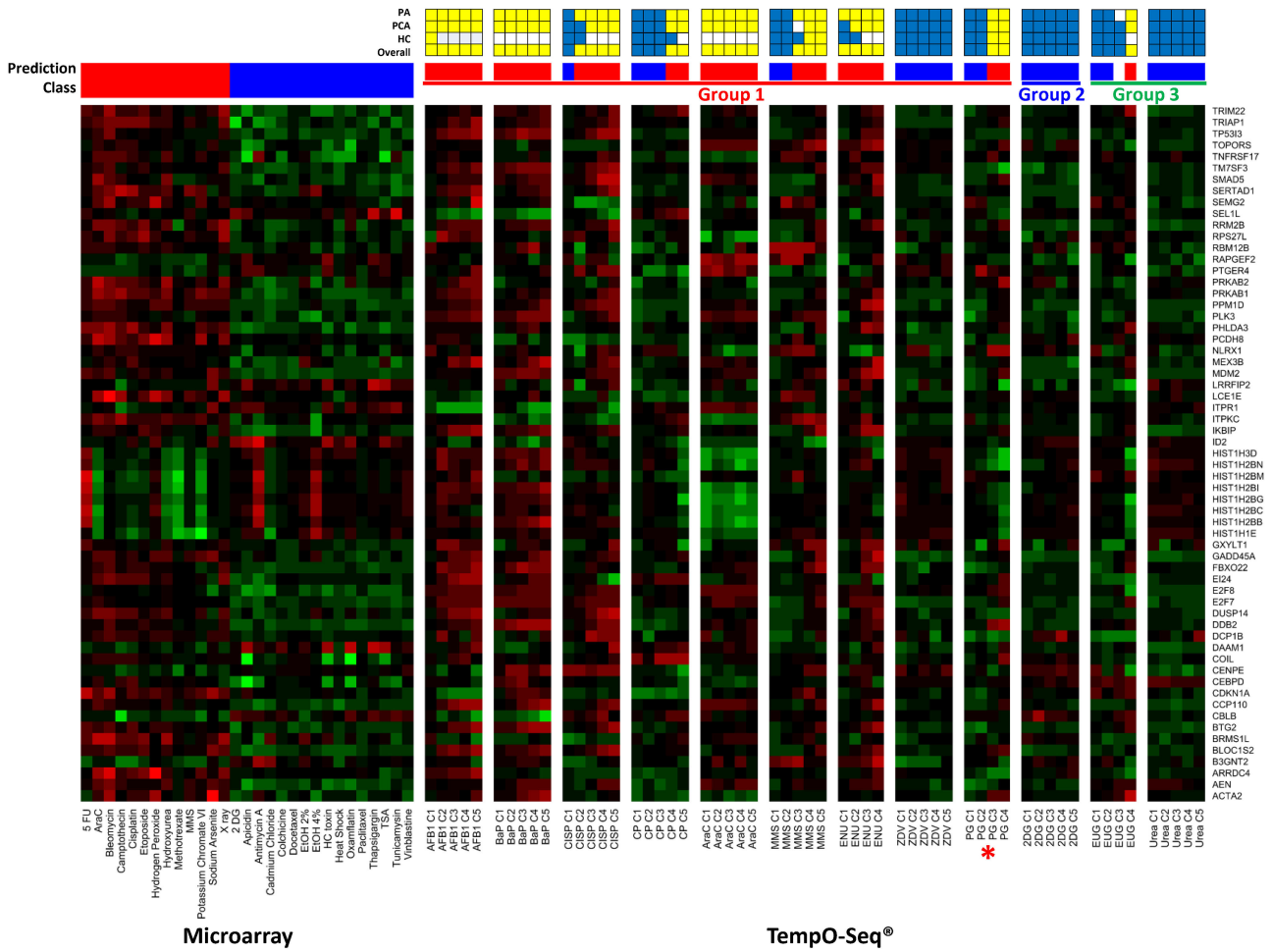

FIGURE 3 | TGx-DDI classification results are depicted using TempO-Seq® gene expression technology for the 12 test chemicals. The heatmap on the left shows the gene expression responses of the TGx-DDI biomarker genes for the 28 reference compounds used to generate the biomarker using DNA microarray analysis in TK6 cells. The test chemicals assessed using TempO-Seq® gene expression technology in human HepaRG™ cells are shown in the subsequent heatmaps (columns). Gene Symbols corresponding to the GenBank accession numbers for the TGx-DDI biomarker genes are shown on the right. The color scale specifies fold changes relative to control: up-regulated genes are red, down-regulated genes are green, and genes exhibiting no changes relative to controls are black. Overall calls for all treatment conditions are shown using red (DDI) and blue (non-DDI) bars above each heatmap. Chemical groupings from Kirkland et al. (57, 58) include Group 1 DDI chemicals (red), a Group 2 non-DDI chemical (blue), and Group 3 potentially misleading positive chemicals (green). DDI chemical abbreviations: aflatoxin B1 (AFB1), benzo[a]pyrene (BaP), cisplatin (CISP), cyclophosphamide (CP), cytosine arabinoside (AraC), methyl methanesulfonate (MMS), N-ethyl-N-nitrosourea (ENU), zidovudine (ZVD), and propyl gallate (PG; *included with Group 1 chemicals). Non-DDI chemical abbreviations: 2-deoxy-D-glucose (2DG), eugenol (EUG), and Urea. The grids above the heatmaps indicate the results of the three different TGx-DDI analyses: Probability Analysis (PA, based on nearest shrunken centroid analysis), Principal Component Analysis (PCA) and Hierarchical Clustering (HC). The overall call is DDI if any one of these analyses yields a DDI call. Yellow boxes indicate a positive DDI classification, blue denotes a negative non-DDI classification and white signifies an unclassified response (i.e., does not yield a DDI or non-DDI call).

% tail DNA for ENU, MMS, and ZDV. Exposure to non-DDI (Group 2) and misleading positive compounds (Group 3) did not cause increases in DNA SSBs in HepaRG™ cells even in the presence of overt cytotoxicity.

TempO-Seq® Analysis for TGx-DDI Biomarker Classification

TempO-Seq® S1500+ sequencing was conducted for the purposes of classifying the test chemicals as DDI or non-DDI using the TGx-DDI genomic biomarker. None of the negative assay controls (1X TempO-Seq® Lysis buffer, no lysates) exceeded 1,200 mapped read counts and the positive assay controls (Human Reference Total RNA and Human Brain Total RNA) showed Pearson correlation coefficients between the replicates that were >0.98 for all pairwise comparisons. The outlier analysis (described above) resulted in the removal of two samples (one replicate each of CISP C2 and EUG C4). Thus, the final sample size was $n = 4$, except for CISP C2 and EUG C4 that had an $n = 3$.

Three independent analyses, including NSC PA, PCA, and HC were considered in the overall TGx-DDI classification. **Figure 3** is a heatmap that represents the TGx-DDI predictions for all

TABLE 3 | Summary of CometChip® analysis and TempO-Seq® TGx-DDI classification results for 12 test chemicals.

	CometChip® (DNA damage)					TGx-DDI classification (gene expression)				
Group 1 chemicals	**C1**	**C2**	**C3**	**C4**	**C5**	**C1**	**C2**	**C3**	**C4**	**C5**
Aflatoxin B1	-	-	-	-	-	+	+	+	+	+
Benzo[a]pyrene	-	-	-	+	+	+	+	+	+	+
Cisplatin	-	-	-	+	+	-	+	+	+	+
Cyclophosphamide	-	-	-	-	+	-	-	-	+	+
Cytosine arabinoside	-	+	+	+	+	+	+	+	+	+
Methyl methanesulfonate	-	+	+	+	+	-	-	+	+	+
N-Ethyl-N-nitrosourea	-	-	+	+	X	+	+	+	+	X
Zidovudine (azidothymidine)	-	+	+	+	+	-	-	-	-	-
Propyl gallate	-	-	-	-	X	-	-	+	+	X
Group 2 chemicals	**C1**	**C2**	**C3**	**C4**	**C5**	**C1**	**C2**	**C3**	**C4**	**C5**
2-Deoxy-D-glucose	-	-	-	-	-	-	-	-	-	-
Group 3 chemicals	**C1**	**C2**	**C3**	**C4**	**C5**	**C1**	**C2**	**C3**	**C4**	**C5**
Eugenol	-	-	-	-	X	-	-	U	+	X
Urea	-	-	-	-	-	-	-	-	-	-

C1 (lowest concentration tested) to C5 (highest concentration tested); X = overtly cytotoxic (< 40% cell viability). For the CometChip® data, + indicates statistically significant increase in DNA damage ($p < 0.05$), as measured using the median % tail DNA and - represents no significant DNA damage. For the gene expression data, + represents a DDI classification, - represents a non-DDI classification, and U indicates a sample that was unclassified using the TGx-DDI genomic biomarker (TempO-Seq® data).

12 test chemicals using NSC PA. **Supplementary Figures 2A–L** depicts the PCA results (panel i) and the HC results (panel ii). If a test chemical had a positive call in one or more analyses, it was predicted to be DDI; whereas, a chemical that had a negative call in all three analyses was classified as non-DDI. The TGx-DDI biomarker accurately classified eight out of nine DDI chemicals. Four of the nine DDI compounds, AFB1, BaP, AraC, and ENU, were classified as DDI at all five concentrations tested. CISP classified as DDI at the top four concentrations; MMS classified as DDI at the three highest concentrations; and CP and PG were both predicted to be DDI at top two concentrations tested (C5 was overtly cytotoxic so gene expression analysis was not conducted for the highest concentration of PG). ZVD was the only DDI compound that misclassified as non-DDI at all concentrations tested.

The TGx-DDI biomarker correctly classified 2DG, a non-DDI (Group 2) chemical, as non-DDI at all concentrations tested (**Figure 3**). For Group 3 chemicals (EUG and Urea), which are potentially misleading positives according to Kirkland et al. (57, 58), only urea classified as non-DDI at all concentrations tested (**Figure 3**). EUG classified as non-DDI at the two lowest concentrations, was unclassified at C3, but rendered a positive DDI classification at C4, the highest concentration tested (C5 was overtly cytotoxic, thus, gene expression analysis was not conducted for the highest concentration).

An overview of the CometChip® and TGx-DDI outcomes is shown in **Table 3**. Overall, measurements of DNA damage analyzed by CometChip® were concordant with TGx-DDI classification results for eight test chemicals. Four test chemicals rendered discordant results: AFB1, PG, and EUG yielded negative CometChip® results but positive TGx-DDI calls; and ZDV was positive by CometChip® but negative with TGx-DDI. By combining the CometChip® assay and the TGx-DDI biomarker (i.e., positive in one assay = positive; negative in both = negative), 11 of the 12 test chemicals were accurately classified.

BMC Analysis of CometChip® Data and TGx-DDI Biomarker Genes

BMC analysis is used to mathematically model the concentration-response curves to determine the concentration at which a predefined increase above controls occurs for potency ranking purposes. BMC modeling was conducted to derive BMCs for both apical and transcriptional endpoints and were denoted as follows: BMC CometChip® (BMC_{CC}) and BMC TGx-DDI biomarker genes (BMC_{TGx}). The BMR used was 1SD for both BMC_{CC} and BMC_{TGx}. BMCL and BMCU were also calculated, and these are referenced in the same manner (i.e., $BMCL_{CC}$ and $BMCU_{CC}$; $BMCL_{TGx}$ and $BMCU_{TGx}$, respectively). Two strategies were used to calculate transcriptomic BMCs (i.e., the NTP's approach to genomic dose-response modeling and a bootstrap method); note that only the bootstrap method allowed for the calculation of the 95% confidence intervals for the TGx-DDI gene set (CIs; i.e., distance between the $BMCL_{TGx}$ and $BMCU_{TGx}$). Comparison of calculated BMC values and confidence limits for CometChip® and the TGx-DDI biomarker genes (both methods) are shown in **Table 4**, in addition to the number of TGx-DDI biomarker genes that could be modeled and the ratio of BMC_{TGx}/BMC_{CC} for comparison of median % tail DNA and transcriptomic BMC values.

Of the seven DDI compounds that could be modeled for the CometChip® data, BaP, CISP, AraC, and MMS were the most potent genotoxicants in HepaRG™, with BMC_{CC} of 1.7, 8.5,

TABLE 4 | Comparison of benchmark concentrations for CometChip® (BMC_{CC}) and TGx-DDI biomarker genes (BMC_{TGx}).

	CometChip® BMC	TGx-DDI median BMC		TGx-DDI bootstrap median BMC		
	Median BMC_{CC} ($BMCL_{CC}$ - $BMCU_{CC}$)	#TGx-DDI genes modeled	Median BMC_{TGx}	#TGx-DDI genes modeled	Median BMC_{TGx} ($BMCL_{TGx}$ - $BMCU_{TGx}$)	Ratio BMC_{TGx}/BMC_{CC}
Group 1						
Aflatoxin B1	n.m.	26	2.9	53	2.7 (1.7–5.3)	-
Benzo[a]pyrene	1.7 (1.4–2.3)	15	0.56	50	0.68 (0.36–1.5)	0.39
Cisplatin	8.5 (6.8–11.2)	19	4.5	54	5.4 (2.5–11.5)	0.63
Cyclophosphamide	8,079 (6,093–9,670)	10	4,808	52	4,949 (2,086–8,408)	0.61
Cytosine arabinoside	12.1 (9.0–17.9)	9	16.6	47	12.9 (3.4–36.2)	1.07
Methyl methanesulfonate	21.3 (16.1–28.8)	22	67.1	49	76.5 (48.2–115)	3.56
N-Ethyl-N-nitrosourea	536 (389–761)	21	285	50	271 (163–494)	0.50
Zidovudine (azidothymidine)	82.5 (64.0–110)	n.m.	n.m.	n.m.	n.m.	-
Propyl gallate	n.m.	20	273	56	336 (246–443)	-
Group 2						
2-Deoxy-D-glucose	n.m.	n.m.	n.m.	n.m.	n.m.	-
Group 3						
Eugenol	n.m.	17	535	56	529 (382–748)	-
Urea	n.m.	n.m.	n.m.	n.m.	n.m.	-

Chemicals that displayed a statistically significant increase in median % tail DNA were modeled for CometChip® BMC (BMC_{CC}) analysis. Chemicals that resulted in a positive DDI classification using the TGx-DDI biomarker were modeled for TGx-DDI BMC (BMC_{TGx}) analysis. Chemicals that did not fit the criteria to be modeled for BMC_{CC} or BMC_{TGx} were not modeled (n.m.). TGx-DDI Median BMCs were calculated using the criteria and strategy outlined in the National Toxicology Program's Approach to Genomic Dose-Response Modeling (72). TGx-DDI Bootstrap Median BMCs were calculated using a bootstrap method to allow lower and upper confidence intervals to be calculated.

12.1, and 21.3 μm, respectively. BMC_{CC}'s for ZDV and ENU were 82.5 and 536 μm, respectively; whereas, the data suggest that CP was the least potent DDI compound in HepaRG™ cells with a BMC_{CC} of 8079 μm. Potency ranking for SSBs was thus BaP > CISP > AraC > MMS > ZDV > ENU > CP > (negative in CometChip® – AFB1, PG, 2DG, EUG, Urea).

BMC_{TGx} analysis for DDI test chemicals with a positive TGx-DDI classification was conducted using two different methods. Both the median BMC_{TGx} and the bootstrap median BMC_{TGx} resulted in very similar potency rankings of the DDI chemicals (**Table 4**). Of the eight DDI compounds that could be modeled for the TGx-DDI data, BaP, AFB1, CISP, AraC, and MMS were the most potent genotoxicants in HepaRG™, with BMC_{TGx} (bootstrap median) of 0.68, 2.7, 5.4, 12.9, and 76.5 μm, respectively. BMC_{TGx} for ENU and PG were 271 and 336 μm, respectively. CP was the least potent DDI compound with a BMC_{TGx} of 4949 μm. For the median BMC_{TGx}, the potency ranking was as follows: BaP > AFB1 > CISP > AraC > MMS > PG > ENU > EUG > CP > (TGx-DDI negative – ZDV, 2DG, Urea); whereas, the ranking of ENU and PG were reversed for bootstrap median BMC_{TGx} (i.e., BaP > AFB1 > CISP > AraC > MMS > ENU > PG > EUG > CP; **Table 4**). The number of TGx-DDI biomarker genes (64 in total) that fit models ranged from 9 (AraC) to 26 (AFB1) using the median BMC approach, but increased substantially from 47 (AraC) to 56 (PG and EUG) using the bootstrap median BMC approach (**Table 4**).

We then directly compared chemicals that were positive in both assays and could be fit to BMC models (i.e., 6 of the 12 chemicals). Remarkably, when comparing chemicals that could be modeled for both CometChip® and transcriptomic endpoints (bootstrap method), the chemical ranking was identical and the ratios of BMC_{TGx}/BMC_{CC} were within 4-fold, ranging from 0.4 to 3.6 (**Table 4** and **Figure 4**). BaP, CISP, CP, and ENU had marginally lower BMC_{TGx} than BMC_{CC}; whereas, the BMC_{TGx} and BMC_{CC} were virtually the same for AraC. MMS was the only test chemical with a lower BMC_{CC} than BMC_{TGx}, and this is consistent with the fact that MMS is the only chemical that is primarily repaired by the base excision repair (BER) pathway; see discussion (**Table 4** and **Figure 4**). The confidence intervals on the BMC_{TGx} are larger than the BMC_{CC}. Overall, the BMC_{CC} and the BMC_{TGx} are highly correlated and result in the same chemical rankings for both the CometChip® assay and the transcriptomic TGx-DDI biomarker assay.

DISCUSSION

To address twenty-first century toxicology needs, an efficient and accurate genotoxicity testing paradigm is urgently required to assess the expanding backlog of data poor chemicals in need of genotoxic evaluation. A high-throughput, integrated test approach that pairs apical and mechanistic data in a human-relevant cell model with metabolic capabilities would be beneficial to address this requirement. TGx biomarkers are useful for this purpose as they enable rapid extraction of mechanistic data from HTTr data (36), which is more compelling when paired with a measure of DNA damage. To address this need, here we have combined a measure of DNA damage (the CometChip® assay) with the TGx-DDI genomic biomarker in physiologically-relevant human HepaRG™ cell cultures for hazard identification

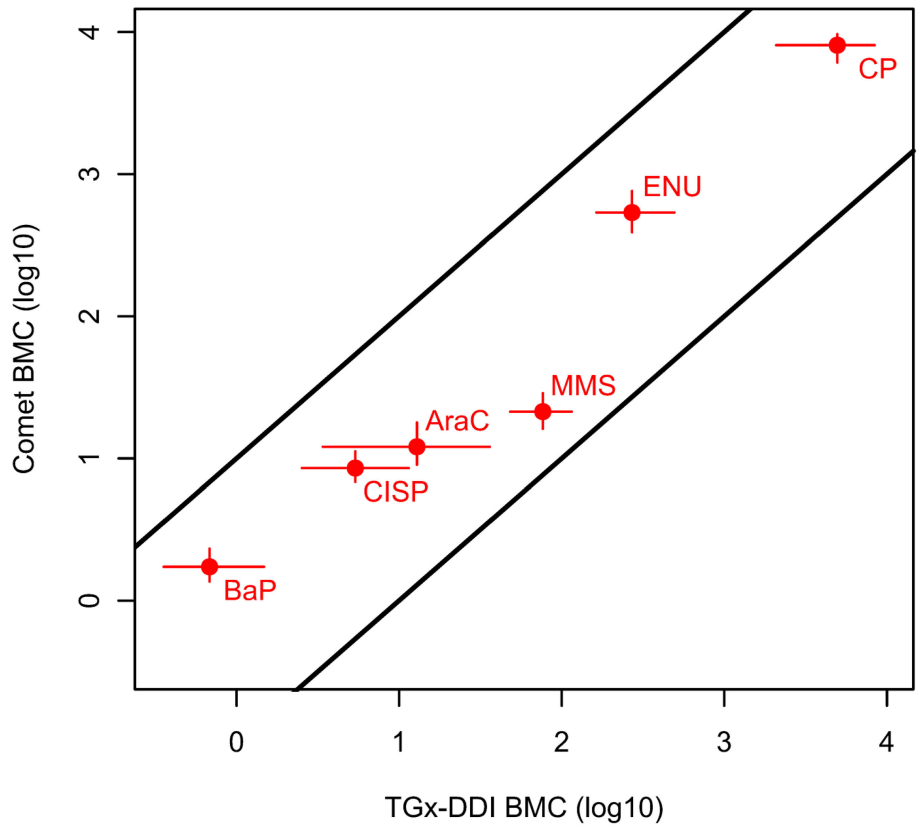

FIGURE 4 | Relationship between the BMCs of the CometChip® data (BMC_{CC}) and the TGx-DDI biomarker genes (BMC_{TGx}) for the test chemicals that yielded positive hazard calls in both tests and could be modeled. Specifically, the BMC_{CC} with a BMR of 1SD with two-sided 95% CIs are shown for the CometChip® vs. median bootstrap BMC_{TGX} (BMR of 1SD) with two-sided 95% CIs for the TGx-DDI biomarker classification endpoint. The BMCs for the agents that were classified as DDI from both approaches were within 10-fold. The two parallel black lines with intercepts of 1 and −1 on the double log10 scale represent a 10-fold deviation from the 1:1 line.

and quantitative analysis of genotoxic potential of DDI and non-DDI chemicals.

We exposed differentiated human HepaRG™ cells using a 3-day daily repeat exposure protocol to 12 test chemicals with varying modes-of-action, including nine DDI (Group 1), one non-DDI (Group 2), and two potentially misleading positives (Group 3). The TGx-DDI transcriptomic biomarker was analyzed with the high-throughput TempO-Seq® platform to establish the value of its integration with the high-throughput CometChip® assay to assess DNA damage. Although each assay had merit on its own, integration of these genotoxicity tests correctly classified all of the DDI agents (Group 1), the non-DDI agent (Group 2), and identified one of two Group 3 chemicals (i.e., "misleading" positive) as non-DDI. BMC modeling of both endpoints revealed identical potency rankings for SSBs compared to transcriptional changes (i.e., BMC_{TGx}/BMC_{CC} ratios were within 4-fold). We conclude that integration of the CometChip® assay with the TGx-DDI genomic biomarker in HepaRG™ cells provides an effective and higher-throughput approach to genotoxicity testing to accurately identify and prioritize chemicals that cause DNA damage and to evaluate their potency. Below we discuss the concordant and discordant results in the context of the complementarity of these assays.

We first explored the concordance of hazard calls made using the CometChip® assay with the TGx-DDI transcriptomic biomarker. Eight of 12 test chemicals yielded concordant hazard calls (i.e., caused SSBs and classified as DDI, or did not cause SSBs and classified as non-DDI). Of the nine DDI chemicals, six produced concordant results albeit at differing concentrations: BaP, CISP, CP, AraC, MMS, and ENU. Of these, the TGx-DDI biomarker measured using TempO-Seq® was somewhat more sensitive at detecting DNA damage for five of the six chemicals. Specifically, it classified chemicals as DDI at lower concentrations than at which DNA damage was observed using the CometChip® assay (MMS was the only exception). This is consistent with our BMC analysis, where MMS was the only chemical with a BMC_{TGx}/BMC_{CC} ratio >1. This is interesting as MMS is the only test chemical included in this study where DNA damage is almost exclusively repaired by means of Base Excision Repair (BER) (79). BER enzymes eliminate damaged bases, which result in persistent SSBs as requisite DNA repair intermediates. The alkaline comet assay does not directly detect damaged base lesions, rather they are indirectly measured when the DNA repair enzymes create

strand breaks in the repair process. This is in contrast to the highly coordinated Nucleotide Excision Repair (NER) pathway that is extremely efficient in repairing damage and therefore minimizes the detectable SSB repair intermediates (55). Thus, there was a large degree of concordance in genotoxicity hazard calls between the assays, with a marginally increased sensitivity of TGx-DDI at lower concentrations.

Conversely, there were three instances of discordant test results across the two assays for the nine DDI chemicals tested: AFB1, PG, and ZDV. AFB1 was negative by CometChip® but had a very strong transcriptional DNA damage response and was classified as DDI using the TGx-DDI biomarker. A negative comet result is not unexpected for AFB1, as it is a genotoxic carcinogen that induces bulky DNA adducts. The alkaline comet assay is best suited for the identification of SSBs, abasic sites, and alkali-sensitive sites (31); it is not very sensitive in the detection of bulky lesions as they do not directly affect DNA migration. Thus, modifications to the standard Comet assay greatly increase sensitivity and therefore help to reliably detect bulky DNA adducts that are actively repaired by means of NER in a highly coordinated fashion (possibly with short-lived NER-induced SSB repair intermediates) (55, 80–83). Specifically, co-exposure to hydroxyurea and AraC traps NER intermediates, allowing for SSB repair intermediates to persist, which greatly improves the sensitivity of the assay for bulky lesions [e.g., (55)]. It is also possible that DNA damage may have been detectable with the CometChip® assay following AFB1 exposure had we used a higher test concentration that reduced cell viability to the 40% target, as a modest increase was seen with other adduct-forming chemicals (e.g., BaP and CP) that did achieve this level of cytotoxicity. It is also important to consider the dynamics of repair and metabolism; it is possible that analysis at a different time point or in a different human cell line could yield different findings. Distinct biotransformation properties and genotoxic responses are associated with both cell line and time, which can influence the detection capabilities of the assay (75, 84). Nonetheless, we note that the TGx-DDI biomarker identified AFB1 as a strong positive, supporting the complementarity of these assays.

Conflicting responses were also observed for PG, an additive used to prevent oxidation (negative CometChip® and positive TGx-DDI) (78, 85). PG is positive in the Ames assay with S9 metabolic activation (59) and induces MN and chromosomal aberrations *in vitro* and *vivo* (60, 86). Thus, the TGx-DDI biomarker correctly classified PG as DDI lending support to the removal of PG from Kirkland et al. (58) Group 3 chemical list. Some antioxidant chemicals, including PG, promote the generation of reactive oxygen species at elevated concentrations (85, 86). Thus, it is possible that we may have detected oxidative DNA damage following PG exposure using a formamidopyrimidine-DNA glycosylase (Fpg)-modified Comet assay, as this lesion-specific enzyme can convert undetectable base lesions caused by oxidative DNA damage into detectable SSBs (31).

Finally, ZDV also yielded discordant outcomes; in this case, a strong DNA damage response by CometChip® was observed with a non-DDI prediction using the TGx-DDI biomarker. This result is plausible and not unexpected as ZDV, also known as azidothymidine, is an anti-HIV medication that belongs to a class of nucleoside analog reverse-transcriptase inhibitors (87), which can dampen the gene expression response (88, 89). Indeed, visual inspection of the heatmap of TGx-DDI genes reveals a broad decrease in transcript levels following ZDV exposure (**Figure 3**).

These discordant results highlight the fact that a single *in vitro* genotoxicity test is not likely to detect all DDI compounds due to the vast array of genotoxic MoAs and the limitations inherent to specific genotoxicity assays. However, when a standardized DNA damage test (i.e., the Comet assay) is paired with a transcriptomic biomarker for DNA damage (i.e., the TGx-DDI biomarker), this built-in test redundancy helps to ensure correct classification and indicates when further follow-up may be necessary to further assess certain chemicals. Indeed, Allemang et al. (90) compared classical and twenty-first century genotoxicity tools (*in vitro* MN, ToxTracker assay, and genomics-based methods including TGx-DDI) and found that no single test correctly classified all genotoxicants when used in isolation; however, the ability to identify genotoxicants improved dramatically when the *in vitro* MN assay was combined with another predictive test such as the TGx-DDI biomarker. They determined that a "fit for purpose" approach was required to combine the appropriate assays to maximize the predictive capacity of the tests for genotoxicity assessment.

The TGx-DDI transcriptomic biomarker was originally developed to distinguish DDI from non-DDI compounds to aid in the interpretation of positive *in vitro* genotoxicity outcomes. In our previous work, Li et al. (36) demonstrated that the TGx-DDI biomarker correctly identified nine out of 10 chemicals classified as having "irrelevant positive" *in vitro* chromosome damage results. In this study, there are two potentially misleading positive chemicals from the Group 3 list: EUG and Urea. Group 3 chemicals *should* test negative, but have been reported to induce gene mutations, chromosomal aberrations, or MN, often at high concentrations or high levels of cytotoxicity (57, 58). Urea rendered negative results (i.e., no SSBs and non-DDI classifications) at all concentrations tested for both assays. However, this was not the case for EUG. EUG is a naturally occurring phenolic molecule found in plants (**Table 1**) (91, 92). While it did not cause any detectable SSBs using the CometChip® assay herein, exposure to EUG resulted in a DDI classification at the highest non-cytotoxic concentration (C4) with TGx-DDI. Although EUG is generally negative for genotoxicity endpoints in p53-competent cells (86), it has tested positive in the mouse lymphoma assay and for chromosomal aberrations at high levels of cytotoxicity – it has been hypothesized that these levels of exposure may overwhelm detoxification leading to positive results (58, 93). The highest concentration of EUG analyzed for TGx-DDI was very close to the cytotoxicity threshold of 60% (56% for EUG C4); thus, it seems that high levels of cytotoxicity may be a plausible reason for the misclassification of EUG herein. Moreover, EUG was tested at a high concentration for C4 (1250 μm), which may have contributed to the misleading positive TGx-DDI classifications at this test concentration, as it may have depleted the detoxification potential of the cells, leaving EUG to cause

primary DNA lesions in some cell types (91). It is possible that the discordant observations for EUG are a result of the different assay sensitivities. Alternatively, it is possible that EUG is exerting a genotoxic effect *via* a different MoA (i.e., a genotoxic mechanism that does not lead to SSBs), which results in a positive TGx-DDI classification in the absence of SSBs. Based on our results and those of the aforementioned studies, we speculate that the discordant results obtained for EUG are in fact relevant and thus require further analysis to explore the DDI potential of this chemical at high concentrations and/or levels of cytotoxicity.

The field of genetic toxicology is shifting toward more quantitative analyses of genetic toxicology data for potency assessments (94–97). Previous work has shown that transcriptional PODs are well-aligned with apical PODs (98–101). Moreover, Bemis et al. (102) demonstrated the correlation between *in vitro* and *in vivo* BMDs for flow cytometric micronucleus data and suggested that the clastogenic potential of a chemical can be calculated from animal studies or cell-based models of chromosome damage. Our previous work in human TK6 cells also demonstrated the concordance of BMC_{MN} and BMC_{TGx} (i.e., within 10-fold) following exposure to three chemicals (68). Herein, we applied BMC modeling to the 12 test chemicals to compare potencies using the CometChip® assay vs. the TGx-DDI biomarker. The BMC_{TGx} values calculated were: (1) the TGx-DDI median BMC, and (2) the TGx-DDI bootstrap median BMC. The bootstrap median BMC method allowed us to model a much higher number of TGx-DDI biomarker genes (e.g., 9 to 26 genes modeled for median BMC method vs. 47 to 56 for the bootstrap median BMC method). It also allowed us to generate 95% confidence intervals for the BMC_{TGx} values (i.e., $BMCL_{TGx}$ and $BMCU_{TGx}$), which is particularly useful for comparing chemical potency rankings. However, given that the concentration ranges differed for the test chemicals in this study, the BMCs were primarily used to compare each chemical's response across the two assays (i.e., BMC_{CC} vs. BMC_{TGx} for each chemical) and BMC comparisons within each assay must be interpreted with caution. For chemicals that had a positive response in both assays, we observed a good correlation between the BMC_{CC} and the BMC_{TGx} in that the ratio of BMC_{TGx} (bootstrap method)/BMC_{CC} was between 0.39 and 3.6 for BaP, CISP, CP, AraC, MMS, and ENU. Of the six chemicals that were modeled for both methodologies, the BMC_{TGx} was more sensitive for four of the chemicals (i.e., BaP, CISP, CP, ENU); the BMCs were virtually identical for AraC, and the BMC_{CC} was more sensitive for MMS. However, it should be noted that the lower BMC_{TGx} values were offset by larger confidence intervals on the TGx-DDI biomarker BMCs, which is expected as this is a composite biomarker that includes many gene BMCs. Nonetheless, chemical rankings are identical (i.e., the ranking from lowest to highest BMC) for the CometChip® assay vs. the TGx-DDI biomarker using the bootstrap method (e.g., BaP > CISP > AraC > MMS > ENU > CP). This study provides further experimental evidence to support the use of BMCs as transcriptional points of departure since they are highly predictive of apical PODs.

In this study, the TGx-DDI assay was conducted using high-throughput targeted RNA-sequencing (TempO-Seq®) to improve the throughput, accuracy, and dynamic range of the gene expression analysis directly from HepaRG™ cell lysates, which also eliminates the requirement to extract RNA thereby improving the efficiency (103, 104). When HTTr is used in combination with a metabolically competent human cell line, such as HepaRG™ cells, it greatly decreases the time and cost required to assess a chemical (no additional test in the presence of S9 needed), while improving the human relevance of this NAM. Beyond the TGx-DDI classification, rich mechanistic data from the transcriptomic data are available for further mining. For example, standard pathway analyses can be applied to explore additional key events and other biomarkers can be analyzed in the same data sets [e.g., we have recently developed the TGx-HDACi transcriptomic biomarker; (105)]. One caveat is that chemicals that inhibit transcription are not amenable to analysis by transcriptomics, which can lead to misclassification (e.g., ZDV, a nucleoside transcriptase inhibitor, misclassified at all concentrations). However, our work and the work of others [e.g., (106, 107)] demonstrate that the use of transcriptomic biomarkers provides a rapid and non-subjective approach to the extraction of information about key toxicological events.

In summary, we demonstrate the potential of a new test paradigm that integrates the TGx-DDI biomarker with the high-throughput CometChip® assay. We validate performance by HTTr profiling in the physiologically-relevant HepaRG™ cell model. Concentration-response modeling for the two tests established the concordance of BMCs for DNA SSBs measured using the CometChip® assay and transcriptional changes in TGx-DDI biomarker genes. This is another step in accomplishing a more integrated genotoxicity testing strategy to derive mechanistic information to better inform human health risk assessment in a higher-throughput manner.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in the NCBI Gene Expression Omnibus under accession number GSE171360. Data are available for download at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE171360.

AUTHOR CONTRIBUTIONS

CY, JB, AW, MM, CS, LR, SF, and BE were involved in project conception, in the development of the analytical approach, and in the data interpretation. CY, JB, and CS designed the study, in consultation with the other authors. CY obtained funding for the project. LR and CS conducted the HepaRG™ exposures, cell viability and CometChip® assays. JB conducted the TempO-Seq® gene expression experiments and prepared the manuscript with important intellectual input from CY, LR, CS, SF, and BE. MM was responsible for the read alignment and bioinformatics analysis of the sequencing data. AW conducted the statistical analyses and prepared some of the figures. RG was instrumental in the establishment of the TempO-Seq® methodology and analysis in the laboratory. JB, CY, AW, MM, RG, and JB had

complete access to the study data. All authors read, reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2021.694834/full#supplementary-material

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