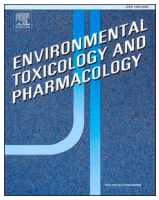
Environmental Toxicology and Pharmacology 105 (2024) 104328

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

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



DNA damage in workers exposed to pigment grade titanium dioxide (TiO_2) and association with biomarkers of oxidative stress and inflammation

Sa. Bonetta [a,*,1], M. Macrì [b,1], M. Acito [c], M. Villarini [c], M. Moretti [c], Si. Bonetta [b], D. Bosio [a], G. Mariella [a], V. Bellisario [a], E. Bergamaschi [a,2], E. Carraro [a,2]

[a] Department of Public Health and Pediatrics, University of Torino, Via Santena 5 Bis, 10126 Torino, Italy
[b] Department of Life Sciences and Systems Biology, University of Torino, Via Accademia Albertina 13, 10123 Torino, Italy
[c] Department of Pharmaceutical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy

ARTICLE INFO

Keywords:
Buccal micronucleus cytome assay
Comet assay
DNA damage
Oxidative stress
Paint production
Pigment-grade TiO_2

ABSTRACT

The present study was aimed at investigating DNA damage, micronuclei frequency and meta-nuclear alterations in buccal cells of workers involved in pigment-grade TiO_2 production (15 exposed and 20 not-exposed). We also assessed associations of genotoxicity biomarkers with oxidative stress/inflammatory biomarkers in urine and exhaled breath condensate (EBC), as well as possible associations between biomarkers and reported respiratory symptoms. In spite of compliance with TiO_2 Occupational Exposure Limits, results showed increased direct/oxidative DNA damage and micronuclei frequency in exposed workers. Genotoxicity parameters were associated with oxidative stress/inflammation biomarkers in urine and EBC, thus confirming that TiO_2 exposure can affect the oxidative balance. Workers with higher genotoxic/oxidative stress biomarkers levels reported early respiratory symptoms suggesting that molecular alterations can be predictive of early health dysfunctions. These findings suggest the need to assess early health impairment in health surveillance programs and to address properly safety issues in workplaces where TiO_2 is handled.

1. Introduction

Titanium dioxide (TiO_2) is a white, incombustible, odourless powder used as white pigment because of its brightness properties (Pelclova et al., 2017b). TiO_2 is used in many different activities, such as paint production, medicine, agriculture, food and cosmetic industries (Baranowska-Wójcik et al., 2020). Despite the promising commercial opportunities, scientific literature showed that TiO_2 can induce adverse biological effects. Titanium dioxide (CAS No 13463–67–7) is classified in hazard class Carcinogenicity, category 2, by the European Chemical Agency (European, 2021) and by the International Agency for Research on Cancer (IARC) (IARC, 2010) - Class 2B - together with the hazard statement H351 – Suspected of causing cancer (inhalation), if present in the form of dust containing at least 1% of particles with an aerodynamic diameter of 10 μm or less. Moreover, the National Institute for Occupational Safety and Health (NIOSH) assigned as potential occupational carcinogen the inhaled ultrafine TiO_2 particles (NIOSH, 2011).

Several *in vitro* studies evaluated the effects of TiO_2 particles of different sizes highlighting that the physicochemical characteristics (i.e. size, crystallinity, shape and coating) could determine different adverse biological effects such as inflammation, cytotoxicity, genotoxicity and cell apoptosis (Gea et al., 2019; Park et al., 2014). Moreover, the results obtained *in vivo* showed the increase of the incidence of lung tumours in rats at high-dose of inhalation exposure (Heinrich et al., 1995; Lee et al., 1985; IARC, 2010), although in some studies no differences in tumour development were observed between exposed and not-exposed rats (Muhle et al., 1991; IARC, 2010).

Health effects of TiO_2 represent an important issue especially in occupational environments accounting for the higher workers' exposure than the general population. However, only few studies were carried out to analyse the different potential adverse biological effects of TiO_2 in occupational settings.

By analysing the exhaled breath condensate (EBC) of workers involved in TiO_2 nanoparticles (TiO_2-NPs) production and handling, Pelclova and collaborators found an increased levels of different oxidation biomarkers (e.g. malondialdehyde (MDA), 8-hydroxy-2-

* Corresponding author.
E-mail address: sara.bonetta@unito.it (Sa. Bonetta).
[1] These authors equally contributed to the research.
[2] These authors equally contributed as last authors.

https://doi.org/10.1016/j.etap.2023.104328
Received 27 September 2023; Accepted 22 November 2023
Available online 25 November 2023

deoxyguanosine (8-OHdG), o-tyrosine (o-Tyr)) that could reflect a possible early health effects on the respiratory tract (aPelclova et al., 2016, 2017; bPelclova et al., 2017; Pelclova et al., 2015, 2016b). Moreover, some studies carried out on the same cohort (Buonaurio et al., 2020; Cavallo et al., 2023; Ursini et al., 2021) have found an increase of titanium (Ti) and DNA oxidized bases in post-shift urine samples and the induction of genotoxic and cytotoxic effects in workers' buccal cells and, to a lesser extent, in peripheral lymphocytes of exposed workers. Our previous investigation in a paint production plant revealed an increased oxidative stress markers in EBC of workers exposed to pigment-grade TiO_2 indicative of subtle alterations of lung pathobiology (Bergamaschi et al., 2022).

The potential genotoxicity of TiO_2-NPs *in vitro* has been investigated with the comet assay and the micronucleus test (Wani and Shadab, 2020); these tests represent a useful tool to evaluate the adverse early health effects induced by several pollutants also in occupational settings. The comet assay measures the DNA-strand break in eukaryotic cell lines or in human biological samples (Singh et al., 1988; Tice et al., 2000). A test modification based on the addition of different enzymes (e.g. the formamidopyrimidine DNA glycosylase-Fpg enzyme) allows to obtain further information on the type of effect, such as DNA oxidative stress (Tice et al., 2000). Otherwise, the micronucleus test allows the detection of chromosome loss and chromosome breaks, i.e. a stabilized DNA damage (Fenech, 2000). The buccal micronucleus cytome (BMcyt) assay has become increasingly important since it allows to measure both the frequency of micronuclei and cell proliferation, cytokinesis defect, different stages of apoptosis and cell death in buccal cells. This method was widely used in biomonitoring of subjects exposed to different airborne genotoxic compounds, but the application in occupational settings remains limited (Bolognesi et al., 2013).

Owing to the limited information available about the potential adverse health effects following TiO_2 exposure in occupational setting, especially genotoxic effects, we planned a study aimed at quantifying the primary DNA damage, the oxidative DNA damage, the frequency of micronuclei and meta-nuclear alterations in oral mucosa cells in the same group of workers exposed to pigment grade TiO_2 investigated in a previous study (Bergamaschi et al., 2022). To detect the genotoxic damage, we sampled the cells from oral mucosa, which are directly exposed to airborne harmful substances. This should allow to gather information on early changes occurring before possible systemic effects. Moreover, these cells can be easily collected by non-invasive sampling which is better tolerated by workers involved in the monitoring.

We assessed the existence of a relationship between the results of genotoxicity tests and the level of pro-inflammatory cytokines (TNF-α, IL-10, IL-1ß), surfactant protein D (SP-D), Krebs von den Lungen-6-glycoprotein (KL-6) in EBC and of oxidative stress biomarkers (8-iso-prostane and MDA) in urine of the same cohort of workers previously evaluated (Bergamaschi et al., 2022). To better understand the health significance of such biochemical changes, we also evaluated a possible association between biomarkers and respiratory symptoms recorded with a questionnaire.

2. Materials and methods

2.1. Study design and worker recruitment

The study was approved by the Bioethical Committee of the University of Turin (Protocol number 256219/2019). The use of an informed consent explaining the methods and the objectives of the study was provided.

The study was performed in a company involved in paint production located near Turin, North-West Italy. In this company 35 volunteer workers were recruited: 15 exposed involved in different production areas of the factory and 20 not-exposed workers (controls) belonging to administration, design and marketing office of the same factory.

All male workers that subscribed the informed consent and provided all biological samples (buccal and salivary cells, urine and EBC samples) were involved in the study. These workers were the same considered in our previous work (Bergamaschi et al., 2022).

Information about health status, the presence of acute and chronic illnesses, drug consumption, working history, living environments, smoking status, alcohol and food consumption, physical activity and other lifestyle habits of all participants were collected with a questionnaire.

2.2. Air monitoring

By a site visit, we identified different areas of the factory, corresponding to specific operations: 1) water-based paints and storage; 2) enamels and solvents; 3) quality control laboratory; 4) packaging; 5) warehouse and 6) administrative office. Among these, we selected the areas characterised by greater production activity for environmental sampling (water-based paint system, automatic bin filling, mixing and dispersion), whereas the administrative office was considered for environmental background (outside the production area).

The environmental air sampling strategy, along with the results, are described in detail in our previous publication (Bergamaschi et al., 2022). Briefly, air sampling near the main processing areas was performed with Air Check Touch fixed cyclone head samplers (SKC, PA) equipped with polycarbonate filters (0.2 μm cut-off). Respirable dusts (mg/m^3 8 h-TWA) and respirable TiO_2 ($\mu g/m^3$ 8 h-TWA) were quantified in the different areas. Moreover, the physicochemical characteristics (shape, size, crystalline form and reactivity) of the powders containing TiO_2 handled by the workers were analysed as reported in Bergamaschi et al. (2022). The powders containing TiO_2 were reported to be 100% rutile, i.e. less reactive and hazardous compared to anatase (Bergamaschi et al., 2022).

2.3. Biological sampling for BMCyt assay and comet assay

The exfoliated epithelial buccal cells for BMCyt assay were collected by softly scraping of both cheeks with a toothbrush, after accurate washing of mouth with water. The toothbrush was immediately immersed in Saccomanno fixative (50% ethanol and 2% polyethylene glycol in water) and shaken to allow the epithelial buccal cells detachment from toothbrush. Samples were transported to the laboratory, where they remained for at least 24 h at + 4 °C. Cells were centrifuged (500 x g, 10 min) and washed twice in PBS (+ 4 °C, pH 7.3). Cell suspension was filtered with 100 μm nylon filter and centrifuged. Pellet obtained was resuspended in PBS (+ 4 °C). Cells were then immersed in methanol and glacial acetic acid (1:1) and sent refrigerated overnight at Cytogenetics Laboratory at the Unit of Public Health, University of Perugia, for slide preparation and scoring.

Salivary leucocytes for comet assay were collected with mouth washing with 0.45% sterile saline solution after accurate washing of mouth with water. The content of the mouth was collected in 0.9% sterile saline solution and transported to laboratory (+ 4 °C), and immediately processed.

Samples were centrifuged (1100 x g, 10 min) and pellets were resuspended in PBS (+37 °C). Cell suspension was centrifuged (44 x g, 10 min); supernatant was collected and centrifuged (1100 x g, 10 min). Afterwards, pellet was resuspended in 1 ml of PBS (+ 37 °C). An amount of 2.4×10^4 - 3.0×10^4 was collected and used for comet assay.

2.4. Buccal micronucleus cytome assay (BMCyt assay)

The BMCyt assay was carried out according to Thomas and Fenech (2011). Two slides were prepared for each subject. Cell suspension (~1 $\times 10^5$ cells) was drip onto microscope slides. Slides were fixed 1 min in 50% ethanol and 1 min in 20% ethanol, washed with water and then immersed for 30 min in HCl 5 M. Slides were washed in distilled water and stained with Schiff's Reagent (60 min). Then, slides were

counterstained with 0.2% Light Green (30 s), washed with distilled water, air-dried and mounted with DePex solution and let dry overnight.

Scoring was blinded and a fixed number of differentiated cells (DC) were counted (minimum of 1000 cells/slide for micronuclei and nuclear buds; minimum of 500 cells/slide for all the other parameters) in order to establish frequency of various types of cells. Abnormalities related with cell death and nuclear abnormalities (related to chromosomal instability or DNA damage) were categorised according to the criteria established by Bolognesi et al. (2013). Micronuclei (MN) and nuclear buds (NBUD) were scored to measure chromosomal and DNA damage, respectively. Cell proliferation was assessed with basal cells (BC) count, cytokinesis defect (cytotoxicity) was quantified with binucleated cells count (BNC), apoptosis stages and cell death were considered with condensed chromatin cells (CCC), karyorrhectic (KHC), pyknotic (PYK) and karyolytic (KYL) cells count.

2.5. Comet assay on salivary leucocytes

The comet assay (pH>13) for detection of primary DNA damage was carried out according to Tice et al. (2000) with slight modification (Bonetta et al., 2019).

Briefly, cell suspension (2.4×10^4 - 3.0×10^4 cells) was mixed with 0.7% low melting agarose (LMA) and an aliquot were laid on frosted microscope slides (covered previously with 1% normal melting agarose (NMA) in PBS and left overnight at room temperature) and covered with a coverslip. Slides were left at + 4 °C for 10 min for LMA solidification. Then another layer of 70 μL of LMA was placed and left at + 4 °C for 10 min. Slides were placed at + 4 °C overnight in lysis solution. Afterwards, slides were immersed in electrophoresis buffer (20 min) and undergone to electrophoresis in the same buffer (20 min, 1 V/cm and 300 mA). Subsequently, slides were immersed in neutralization buffer (3 min), fixed (ethanol 70%) and air-dried overnight.

The modified version of comet assay (+ formamidopyrimidine-DNA glycosylase enzyme Fpg) for detection of oxidative DNA damage was carried out according to Bonetta et al. (2009) with slight modification (Gea et al., 2019). Briefly, the test was performed as previously described but, after lysis, the slides were washed with Fpg buffer (5 min, three times). Then, each slide was exposed at 1 unit of Fpg enzyme or Fpg buffer (procedure control slides) (37 °C, 30 min) and undergone to electrophoresis as described before.

For the DNA damage quantification, the slides were stained with ethidium bromide (20 μg/ml) and the percentage of DNA in tail (% of tail intensity) was estimated using a fluorescence microscope (Axioskop HBO 50, Zeiss) furnished with the Comet Assay IV analysis system (Perceptive Instruments, Instem). The DNA damage, expressed as % of tail intensity (%TI), was evaluated in 100 cells for each subject.

The oxidative DNA damage was obtained by subtraction of %TI in procedure control slides from the %TI in enzyme-treated slides.

2.6. Oxidative and inflammatory biomarkers in urine and EBC

As previously described by Bergamaschi et al. (2022), the EBC and a spot sample of urine were collected for the measurement of both oxidative stress and inflammation biomarkers. Several compounds were analysed as biomarkers of early and subclinical effects in EBC (TNF-α, IL-1ß, IL-10, SP-D, KL-6) and in urine (8-isoprostane, MDA), in order to evaluate the inflammatory and oxidative stress profile of the subjects. The levels of biomarkers measured in urine and EBC of workers were reported in our previous study (Bergamaschi et al., 2022). In the present study, the results of these biomarkers were considered to investigate a possible association with the genotoxic and oxidative damage.

2.7. Statistical analysis

All analyses were carried out using the STATA 16.1 software (StataCorp LLC: College Station, TX, USA). The normality of data was checked through the Kolmogorov–Smirnov's test while the Levene's test was used to evaluate the homogeneity of the examined population. As the statistical distribution of the quantitative parameters was found to be non-Gaussian (Kolmogorov-Smirnov's test), non-parametric tests were used to assess between group differences (Mann-Whitney U-test, Spearman's correlations test). Two-sided p value < 0.05 was considered to indicate statistical significance. Association between the presence of respiratory symptoms, DNA damage and oxidative stress biomarkers was investigated by binary logistic regression analysis model, adjusted for smoking habit and working exposure. Results were reported as odds ratios (ORs) with 95% confidence intervals (CIs).

3. Results and discussion

3.1. Environmental airborne dust sampling

The environmental samplings were carried out in the following sampling points: water-based paint area, automatic bin filling area, mixing and dispersion area and administrative office (Table S1).

The highest values of respirable dusts and Ti were observed in the production areas (0.137 mg/m^3 and 0.114 μg/m^3, respectively), whereas the lowest in the administrative office (0.033 mg/m^3 and 0.013 μg/m^3, respectively) (Bergamaschi et al., 2022). These results are in accordance with the trend reported in other studies, although the Ti levels detected in the present study (range of 0.013–0.114 μg/m^3 as the weighted average concentration over an 8 h working time (8 h-TWA) in stationary samples) are lower than those measured in the other occupational settings investigated. For instance, Peclova et al. (2015) reported 0.65 and 0.40 mg/m^3 of median total mass, whereas Ursini et al. (2021) measured an average airborne concentration of 0.16 mg/m^3 and 1.06 mg/m^3 in the respirable and inhalable fractions, respectively. These findings can be attributed to the different occupational scenario studied, the formers being production plants, while the present study was carried out in a paint production company, where the workers' exposure can mainly take place during handling in confined areas where TiO_2 powder is added to the paint. The airborne TiO_2 concentrations of respirable dusts confirm likewise the low contamination of the workplace: the values did not exceed the exposure limit of 2.4 mg/m^3 and 0.3 mg/m^3 recommended by NIOSH for fine (>100 nm) and ultrafine TiO_2 particles, respectively (NIOSH, 2011), nor 10 mg/m^3 (TWA) limit value set by the ACGIH for fine TiO_2 particles (ACGIH, 2001).

Table 1
General characteristics of the cohort investigated. Values are reported as mean ± standard deviation (SD). No differences between the groups were found (Levene's test for homogeneity of variance = not significant). All recruited workers were male.

MAIN GENERAL CHARACTERISTICS OF THE POPULATION INVESTIGATED							
	General Sample		**Exposed (N = 15)**		**Not Exposed (N = 20)**		**Levene's test**
Height (cm) Mean + SD	174 ± 8.1		175 ± 8.9		173 ± 8		0.4
Weight (Kg) Mean + SD	80.7 ± 14.5		85.7 ± 14.5		82.3 ± 13.6		0.8
BMI Mean + SD	26.6 ± 4.1		27.9 ± 3.5		26 ± 4.4		0.5
Age (years) Mean + SD	47.3 ± 11.5		48.7 ± 10.05		45.9 ± 12.18		0.2
Working exposure (years) Mean + SD	13.8 ± 10.9		14.3 ± 10.8		13.5 ± 12		0.4
Smoke habits [N.]	YES 9	NO 26	YES 6	NO 9	YES 3	NO 17	0.1

3.2. Study population

Table 1 summarizes the main characteristics of the study group, distinguished on the basis of TiO_2 exposure.

The two groups resulted homogeneous for their demographic characteristics: indeed, no significant differences were observed, even considering the possible confounding factors, in particular smoking habits. Smokers reported 2–10 cigarettes/day. The association between DNA damage and smoking habits is still controversial. While some studies reported higher values of primary DNA damage in smokers than in non-smokers (Osswald et al., 2003; Glei et al., 2005), other authors showed that smoking habit was not statistically correlated with higher % TI in buccal leucocytes (Russo et al., 2020; McCauley et al., 2008). Furthermore, although some investigations showed that the MN frequency in buccal leucocytes was associated with smoking habits (Villarini et al., 2018) in other works only heavy smokers (>40 cigarettes/day) showed a significant increase of MN (Bonassi et al., 2011a). Different factors may contribute to the discrepancies among the results such as the number of subjects recruited, the number and kind of cigarettes smoked and the contribution of the passive smoking.

3.3. BMCyt assay

The results of BMCyt Assay for chromosomal and DNA damage markers (MN and NBUD), cell proliferation markers (BC and BNC) and cell death/apoptosis markers (CCC, KHC, PYK, and KYL) in workers are summarised in Table 2.

The BMCyt Assay revealed a slightly higher MN frequency in buccal cells of the exposed workers as compared to controls ($p < 0.05$, Mann-Whitney test).

The potential of TiO_2 to induce detectable genotoxic damage by MN increase was observed in several *in vitro* studies performed on different cell lines. Liao et al. (2019) tested TiO_2 particles (10, 30, 50, 100 nm of anatase at 1, 5 and 25 μg/ml) on human umbilical vein endothelial cells (HUVECs) for 24 h, and the results demonstrated an increase of MN frequency confirming that, under the conditions tested, TiO_2 particles can induce a detectable genotoxic damage. Even in the study conducted *in vitro* by Demir et al. (2015) a genotoxic effect of TiO_2 (21 and 50 nm in anatase crystalline form) was observed on human embryonic kidney cells (HEK293) at the highest concentration tested (1000 μg/ml) after 48 h of exposure.

While evidence on the genotoxic effects of TiO_2 *in vitro* are well documented (Wani and Shadab, 2020), studies *in vivo* (animal model) are lacking and inconclusive (Lindberg et al., 2012; Vignardi et al., 2015). Considering the human exposure toTiO_2, the only study available in literature was performed by Ursini et al. (2021), in which the potential genotoxic effect associated with occupational exposure to TiO_2 was evaluated with the BMCyt assay in buccal cells. However, this study was carried out in workers employed in a company producing TiO_2 in which the exposure concentration was higher than that detected in our investigation. However, as in the study of Ursini and collaborators (2021), in the present study a slight but significant frequency of MN was revealed in the buccal cells of the TiO_2 exposed workers than in the controls. This finding shows as a chronic occupational exposure to TiO_2 seems to induce genotoxic effects in buccal cells, even at low level of air TiO_2 concentration. It is possible that a chronic exposure of the workers allowed an uptake of the smallest particles that are able to cross the nuclear membrane even at low doses of exposure, as demonstrated in the study of Geiser et al. (2005). The authors observed inside rat lung epithelial cells and cell organelles, including nucleus, the presence of the particles 24 h after 1-hour inhalation exposure to nanoscale TiO_2. As suggested by Ursini et al. (2020) the particles present in the nucleus can break DNA producing MN during failed reparation process. Despite the slightly higher MN frequency in exposed workers than in not-exposed subjects, no difference for the cell death and apoptosis markers (CCC, KHC, PYK, and KYL) between the two groups was observed. This finding is in agreement with those obtained in *in vitro* studies, where a mild cytotoxic effect induced by TiO_2 was detectable even in presence of statistically significant DNA damage (Gea et al., 2019; Pedrino et al., 2022).

Considering the results of the proliferation markers (BC and BNC), a tendency towards lower number of BC and a slightly higher number of BNC were observed in exposed workers than the controls, even if the difference is not statistically significant (Table 2). This finding could point out an interference in the normal cell cycle leading to a failure of the cytokinesis, probably related to the cytotoxic damage induced by TiO_2 particles in the buccal cells.

Table 2
Results of BMCyt assay and comet assay. Values are reported as mean ± standard deviation (SD). Bold indicates statistically significant values. DC: differentiated cells; MN: micronuclei; NBUD: nuclear buds; BC: basal cells; BNC: binucleated cells; PYK: pyknotic cells; CCC: condensed chromatin cells; KHC: karyorrhectic cells; Kyl: karyolytic cells; %TI: direct DNA damage; %TI ox: oxidative DNA damage.

Genotoxicity assays (BMCyt assay and comet assay ± Fpg)				
	Total	Exposed	Not exposed	Mann-Whitey U test
DC/500 cells Mean + SD	440.6 ± 36.7	449 ± 27	440 ± 36.1	0.6
MN/1000 cells Mean + SD	3.4 ± 1.6	3.2 ± 1.5	2.8 ± 1.5	**0.04**
NBUD/1000 cells Mean + SD	0.02 ± 0.1	0.03 ± 0.1	0.05 ± 0.1	0.7
BC/500 cells Mean + SD	0.01 ± 0.2	0.07 ± 0.2	0.2 ± 0.2	0.1
BNC/500 cells Mean + SD	4.2 ± 1.8	4.5 ± 1.9	4.4 ± 1.8	0.8
PYK/500 cells Mean + SD	0.5 ± 0.6	0.4 ± 0.6	0.3 ± 0.4	0.9
CCC/500 cells Mean + SD	34.7 ± 23.4	30.3 ± 18.6	35.7 ± 22.4	0.6
KHC/500 cells Mean + SD	6 ± 6.4	5.2 ± 5.1	5.4 ± 6.7	0.8
Kyl/500 cells Mean + SD	13.9 ± 10.6	10.8 ± 7.7	13.8 ± 10.2	0.4
%TI Mean + SD	4.6 ± 3	6.4 ± 3.1	3.2 ± 1.9	**0.04**
%TI ox Mean + SD	6 ± 4.9	8.3 ± 4.8	2.9 ± 2.8	**0.03**

3.4. Comet assay

Table 2 also reports the results of the direct DNA damage and oxidative DNA damage on salivary leucocytes of workers evaluated with the comet assay and the Fpg enzyme-modified comet assay, respectively.

The primary DNA damage was significantly higher in the workers exposed to TiO_2 than in the not-exposed subjects ($p < 0.05$, Mann-Whitney test). Moreover, the oxidative DNA damage was higher in the TiO_2-exposed workers as compared to the not-exposed subjects ($p < 0.05$, Mann-Whitney test). Interestingly, on the whole group, both the direct and the oxidative DNA damage resulted statistically associated with Ti-U concentration (Table 3). This association with the Ti-U - a biomarker of exposure – suggests the potential role of TiO_2 exposure in the induction of direct and oxidative DNA damage, occurring even at low doses.

It should be pointed out that the damage detectable by the comet assay is a reversible damage that can still be repaired. Therefore, the results obtained with the comet assay are compatible with the detection of biological effects of low intensity, which seems consistently associated with low environmental concentrations of TiO_2. The presence of DNA damage associated with TiO_2 exposure has been also reported in studies that have used the comet assay both *in vitro* and *in vivo* (Chen et al., 2014; Wani and Shadab, 2020; Ling et al., 2021). Gea et al. (2019),

Table 3
Results of Spearman's correlation between DNA damage parameters monitored in buccal cells and oxidative stress, inflammation and exposure biomarkers monitored in EBC or urine. Bold indicates statistically significant values. %TI: direct DNA damage; %TI ox: oxidative DNA damage; MN: micronuclei; MDA: malondialdehyde; SP-D: surfactant protein D; Ti-U: urinary titanium.

	%TI	%TI ox	MN
%TI	–	**0.525**	0.096
		p = 0.001	p = 0.583
%TI ox	**0.525**	–	0.071
	p = 0.001		p = 0.687
MN	0.096	0.071	–
	p = 0.583	p = 0.687	
MDA	**0.441**	**0.442**	0.069
	p = 0.008	**p = 0.008**	p = 0.694
8-isoprostane	0.291	**0.347**	0.253
	p = 0.090	**p = 0.041**	p = 0.142
TNF-α	**0.455**	-0.234	-0.216
	p = 0.006	p = 0.176	p = 0.213
IL-1ß	**0.505**	**0.482**	0.036
	p = 0.002	**p = 0.003**	p = 0.839
IL-10	**0.446**	0.313	0.168
	p = 0.007	p = 0.067	p = 0.333
SP-D	-0.322	**0.355**	-0.182
	p = 0.063	**p = 0.039**	p = 0.302
Ti-U	**0.435**	**0.523**	-0.055
	p = 0.016	**p = 0.003**	p = 0.755

reported a significant increase of DNA damage in human bronchial epithelium (BEAS-2B) exposed for 24 h to TiO_2 particles (food grade (150 ± 50 nm) and platelets (75 ± 25 nm) in anatase form at 80, 120 and 160 μg/ml); the same trend was also observed in the study of Patel et al. (2017) that tested TiO_2 particles (mixture of anatase and rutile crystalline form with diameter <100 nm at 75 and 125 μM) on human lymphocytes for 24 h. Considering *in vivo* studies, Larsen et al. (2016) observed increased DNA damage (detectable after 24 h) in lung tissue of mice after one-hour exposure to TiO_2 (primary size of 10 ± 3.6 nm at concentration of 271 mg/m^3) through aerosolization.; also the study of Li et al. (2017) showed increased DNA damage in the liver and lungs of mice after three daily intraperitoneal administration of TiO_2 particles (50 mg/kg of 10 nm anatase).

The sensitivity of the comet assay in evaluating the DNA damage related to slight TiO_2 exposure, represents a good prerequisite for its application in environmental and occupational studies dealing with the assessment of the genotoxic potential of hazardous substances (Collins et al., 2023).

To our knowledge, the present study is the first one in which the comet assay was used to assess the DNA damage potentially induced in buccal cells of workers exposed to TiO_2. The only study available in literature in which the comet assay was used to detect genotoxic and oxidative DNA damage in workers producing TiO_2 was performed on lymphocytes from venous blood samples by Cavallo et al. (2023), who found slight, but statistically significant, difference only in the % of comets (DNA damaged cells) between the workers and external controls.

The different results obtained in the present study could be explained with the different biological matrices used. Since the buccal cells represent the first site of contact for TiO_2 occupational exposure, they are more susceptible to DNA fragmentation and oxidation (as suggested also by the study of Ursini et al. (2021).

Moreover, the evidence that TiO_2 can induce respiratory oxidative stress and inflammation in humans was confirmed in several studies using other biomarkers of effect. In Pelclova et al. (2015) the analysis of oxidation biomarkers in the EBC of workers, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and 5-hydroxymethyl uracil (5-OHMeU), pointed out an increased oxidative stress in TiO_2 exposed workers than in not-exposed. Similar results were also reported in the study of Buonaurio et al. (2020), who found an increase of some urinary biomarkers of oxidative stress, such as 8-oxodGuo, 8-oxoGuo and 3-Nitrotyrosine (3-NO_2Tyr) in TiO_2 exposed workers.

3.5. Association between DNA damage, MN frequency and biomarkers of inflammation and oxidative stress

With the aim of evaluating the overall impact of the TiO_2 exposure, the possible association between the DNA damage, the frequency of MN and the biomarkers of oxidative stress and inflammation in EBC and urine samples were investigated. Preliminary findings describing the behaviour of such biomarkers have been reported in details in a previous publication (Bergamaschi et al., 2022).

A positive statistically significant association was observed among different parameters: the direct DNA damage was associated with biomarkers of oxidative stress (i.e. oxidative DNA damage and MDA) and inflammation (i.e. TNF-α, IL-1ß and IL-10) (Table 3). Moreover, the oxidative DNA damage was found to be positively associated with biomarkers of oxidative stress (i.e. MDA and 8-isoprostane) and inflammation (i.e. IL-1ß and SP-D), a potential signal of interstitial lung disease. Conversely, the MN did not show any significant association with these parameters.

These results underline that the exposure even at low level of TiO_2 in occupational settings seems to induce oxidative stress and inflammation detectable in biological matrices (urine, EBC and buccal mucosa). On the other hand, an increased oxidative stress imbalance is one of the main mechanisms underlying TiO_2 induced cell toxicity (in particular the DNA damage).

3.6. Association between biomarkers of effects and respiratory symptoms

In order to investigate a possible relationship with the TiO_2 exposure, the association between respiratory symptoms within the last 12 months as reported in the questionnaire (namely cough, asthma, persistent and/or recurrent nasal disorders and allergies), the DNA damage (direct and oxidative DNA damage), MN frequency, and oxidative stress biomarkers (8-isoprostane and MDA) was analysed. The statistical analysis of these associations with a binary logistic regression analysis model showed that workers with higher values of genotoxic [MN: OR 0.59, 95%CI 1.04–3.49, p = 0.037] and oxidative biomarkers [oxidative DNA damage: OR 0.345, 95%CI 1.05–2.63, p = 0.04) and 8-isoprostane: OR 0.465, 95%CI 1.59–2.93, p = 0.03] also had higher likelihood of develop and report respiratory symptoms (Fig. 1).

The association with respiratory symptoms and oxidative stress is consistent with other studies. Chamitava et al. (2020), found increased 8-isoprostane in people with chronic bronchitis compared to controls, highlighting the link between oxidative stress and respiratory symptoms.

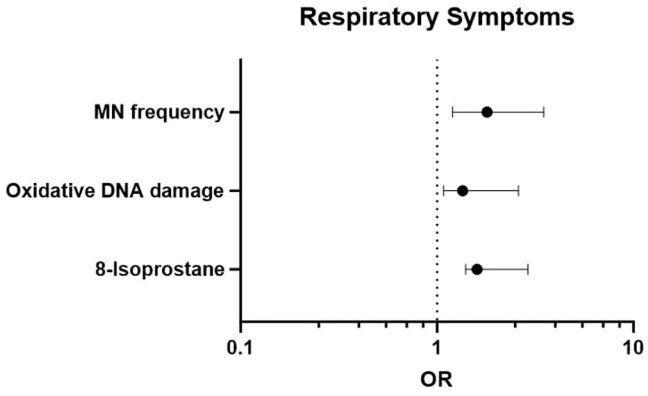


Fig. 1. Association between biomarkers of effects and respiratory symptoms. Binary logistic regression analysis between respiratory symptoms and increased biomarker levels, adjusted for smoke habits and working exposure.

This association was also observed in workers exposed to different kinds of pollutants. In the study of Kargar-Shouroki et al. (2022), the association between lung function impairment and oxidative stress parameters in workers exposed to wood dust was pointed out. Moreover, in the study conducted by Kaushik et al. (2012) in brick kiln workers a significant correlation between oxidative stress parameters and pulmonary dysfunction was observed, which may be due to silica-induced oxidative stress. Also in sewage workers exposed to harmful dust, fumes and gases Shadab et al. (2014) showed an increase of oxidative stress parameters in serum and impairment of lung functions. Furthermore, in the study of Jalilian et al. (2018) an increased presence of respiratory symptoms as well as raised concentrations of oxidative stress biomarkers in workers exposed to a mixture of pesticides was found.

Interestingly, in the present study an association between the presence of respiratory symptoms and the frequency of MN in buccal cells was observed. This is an original finding not reported in other investigations.

Different literature reviews provided evidences that the MN frequency in lymphocytes can be considered as a predictive biomarker of cancer risk in healthy subjects suggesting that increased MN formation is associated with initial event of carcinogenesis (Bonassi et al., 2007; Bonassi et al., 2011b). However, some recent studies showed that MN are generally high in many cancers but also in non-cancer diseases. Moreover, other investigations highlighted that the frequency of MN are positively correlated with inflammation (Durante and Formenti, 2018; McKelvey et al., 2018). These findings confirmed that the MN can be considered not only indicators of chromosome loss and chromosome breaks, but they can also play an important role in the induction of inflammation and diseases (Fenech et al., 2020). Recent discoveries reported that MN generation can lead to both chromosome instability and inflammation and this could clarify the association of MN not only with cancer but also with other diseases that can be mainly triggered by inflammation (Fenech et al., 2021).

Then, the observed association between MN presence and respiratory symptoms could be related to an inflammation process induced by MN formation. This finding could provide a preliminary evidence that MN frequency in buccal cells could be used also as predictive early biomarker of respiratory disease.

4. Conclusion

The objective of this study was to investigate possible early effects in workers exposed to TiO_2 particles in a paint manufacturing company using a non-invasive approach. The use of buccal cells can be a useful tool for assessing exposure risks because oral mucosal cells and salivary leucocytes represent the first site of contact with many airborne pollutants. The results of the comet assay on salivary leucocytes showed an increase in DNA damage (direct and oxidative) in exposed workers compared to not-exposed subjects, although the dusts and TiO_2 levels detected by air sampling were quite low. Similarly, the BMCyt assay revealed a slight increase of MN count in workers exposed as compared to not-exposed.

Micronucleus test and comet assay have proven to be useful biomarkers for assessing genotoxic effects in workers exposed to other pollutants (Leonardi et al., 2020; Collins et al., 2023) and seem to be good indicators of genotoxic effects also in the TiO_2 exposed subjects.

In the present study, the genotoxicity parameters analysed were associated with oxidative stress and inflammation biomarkers showing an early effect on workers' health; a relationship with respiratory symptoms of the analysed subjects was also evident.

The main limitation of the present study is the limited number of workers, making it necessary to assess the health relevance of the above findings in a longer-term perspective and recruit a larger number of subjects.

Finally, the analysis of the results of the studies carried out on this occupational exposure, including the present study, suggests the need of introducing precautionary measures during specific activities and operational phases to further reduce possible risks for workers, considering that there are production realities in which the TiO_2 exposure is higher than that observed in the company investigated in the present work.

Ethics approval and consent to participate

All subjects signed an informed consent before being enrolled. The study was approved by the Bioethical Committee of the University of Turin (Protocol number 256219/2019).

Funding

This study has received funds from Fondazione Cassa di Risparmio di Torino (CRT), bando Erogazioni Ordinarie 2017 (Protocollo: 2017. AI1952. U2223; RF= 2017.2061).

CRediT authorship contribution statement

Sara Bonetta: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization, Validation. **Manuela Macrì**: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization, Validation. **Mattia Acito**: Investigation. **Milena Villarini**: Supervision, Writing – review & editing. **Massimo Moretti**: Supervision, Writing – review & editing. **Silvia Bonetta**: Investigation, Supervision, Writing – review & editing. **Davide Bosio**: Supervision. **Giuseppe Mariella**: Investigation. **Valeria Bellisario**: Formal analysis, Writing – review & editing. **Enrico Bergamaschi**: Conceptualization, Supervision, Resources, Writing – review & editing, Project administration, Funding acquisition. **Elisabetta Carraro**: Conceptualization, Supervision, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank all the workers who participated in the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104328.

References

ACGIH, American Conference of Governmental Industrial Hygienists, 2001, Titanium dioxide. In Documentation of the Threshold Limit Values for Chemical Substances. 7th edition. Cincinnati, OH: American Conference of Governmental Industrial Hygienists.

aPelclova, D., Zdimal, V., Fenclova, Z., Vlckova, S., Turci, F., Corazzari, I., Kacer, P., Schwarz, J., Zikova, N., Makes, O., Syslova, K., Komarc, M., Belacek, J., Navratil, T., Machajova, M., Zakharov, S., 2016. Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO2 (nano) particles. Occup. Environ. Med 73 (2), 110–118. https://doi.org/10.1136/oemed-2015-103161.

aPelclova, D., Zdimal, V., Kacer, P., Komarc, M., Fenclova, Z., Vlckova, S., Zikova, N., Schwarz, J., Makes, O., Navratil, T., Zakharov, S., Bello, D., 2017. Markers of lipid oxidative damage among office workers exposed intermittently to air pollutants

including nanoTiO2 particles, 1 Rev. Environ. Health 32 (1–2), 193–200. https://doi.org/10.1515/reveh-2016-0030.
Baranowska-Wójcik, E., Szwajgier, D., Oleszczuk, P., Winiarska-Mieczan, A., 2020. Effects of titanium dioxide nanoparticles exposure on human health-a review. Biol. Trace Elem. Res 193 (1), 118–129. https://doi.org/10.1007/s12011-019-01706-6.
Bergamaschi, E., Bellisario, V., Macrì, M., Buglisi, M., Garzaro, G., Squillacioti, G., Ghelli, F., Bono, R., Fenoglio, I., Barbero, F., Riganti, C., Marrocco, A., Bonetta, S., Carraro, E., 2022. A biomonitoring pilot study in workers from a paints production plant exposed to pigment-grade titanium dioxide (TiO2), 31; Toxics 10 (4), 171. https://doi.org/10.3390/toxics10040171.
Bolognesi, C., Knasmueller, S., Nersesyan, A., Thomas, P., Fenech, M., 2013. The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay - an update and expanded photogallery. Mutat. Res 753 (2), 100–113. https://doi.org/10.1016/j.mrrev.2013.07.002.
Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28 (3), 625–631 https:// doi: 10.1093/carcin/bgl177.
Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., Kirsh-Volders, M., Knasmueller, S., Zeiger, E., Carnesoltas, D., Cavallo, D., da Silva, J., de Andrade, V.M., Demircigil, G.C., Domínguez Odio, A., Donmez-Altuntas, H., Gattas, G., Giri, A., Giri, S., Gómez-Meda, B., Gómez-Arroyo, S., Hadjidekova, V., Haveric, A., Kamboj, M., Kurteshi, K., Martino-Roth, M.G., Montero Montoya, R., Nersesyan, A., Pastor-Benito, S., Favero Salvadori, D.M., Shaposhnikova, A., Stopper, H., Thomas, P., Torres-Bugarín, O., Yadav, A.S., Zúñiga González, G., Fenech, M., 2011a. The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutat. Res 728 (3), 88–97. https://doi.org/10.1016/j.mrrev.2011.06.005. Epub 2011 Jul 8.
Bonassi, S., El-Zein, R., Bolognesi, C., Fenech, M., 2011b. Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. Mutagenesis 26 (1), 93–100. https://doi.org/10.1093/mutage/geq075.
Bonetta, S., Gianotti, V., Bonetta, S., Gosetti, F., Oddone, M., Gennaro, M.C., Carraro, E., 2009. DNA damage in A549 cells exposed to different extracts of PM(2.5) from industrial, urban and highway sites. Chemosphere 77 (7), 1030–1034. https://doi.org/10.1016/j.chemosphere.2009.07.076.
Bonetta, S., Bonetta, S., Schilirò, T., Ceretti, E., Feretti, D., Covolo, L., Vannini, S., Villarini, M., Moretti, M., Verani, M., Carducci, A., Bagordo, F., De Donno, A., Bonizzoni, S., Bonetti, A., Pignata, C., Carraro, E., Gelatti, U., MAPEC_LIFE Study Group, 2019. Mutagenic and genotoxic effects induced by PM0.5 of different Italian towns in human cells and bacteria: The MAPEC_LIFE study. Environ. Pollut. 245, 1124–1135. https://doi.org/10.1016/j.envpol.2018.11.017.
bPelclova, D., Zdimal, V., Kacer, P., Fenclova, Z., Vlckova, S., Komarc, M., Navratil, T., Schwarz, J., Zikova, N., Makes, O., Syslova, K., Belacek, J., Zakharov, S., 2016. Leukotrienes in exhaled breath condensate and fractional exhaled nitric oxide in workers exposed to TiO2 nanoparticles, 30 J. Breath. Res 10 (3), 036004. https://doi.org/10.1088/1752-7155/10/3/036004.
bPelclova, D., Zdimal, V., Kacer, P., Zikova, N., Komarc, M., Fenclova, Z., Vlckova, S., Schwarz, J., Makeš, O., Syslova, K., Navratil, T., Turci, F., Corazzari, I., Zakharov, S., Bello, D., 2017. Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers. Nanotoxicology 11 (1), 52–63. https://doi.org/10.1080/17435390.2016.1262921.
Buonaurio, F., Astolfi, M.L., Canepari, S., Di Basilio, M., Gibilras, R., Mecchia, M., Papacchini, M., Paci, E., Pigini, D., Tranfo, G., 2020. Urinary oxidative stress biomarkers in workers of a titanium dioxide based pigment production plant, 5 Int J. Environ. Res Public Health 17 (23), 9085. https://doi.org/10.3390/ijerph17239085.
Cavallo, D., Fresegna, A.M., Ciervo, A., Maiello, R., Chiarella, P., Buresti, G., Del Frate, V., Di Basilio, M., Iavicoli, S., Ursini, C.L., 2023. Evaluation of Systemic Genotoxic/Oxidative and Proinflammatory Effects in Workers of a Titanium Dioxide Production Plant. Jul 22;2023 Biomed. Res Int, 7066090. https://doi.org/10.1155/2023/7066090.
Chamitava, L., Cazzoletti, L., Ferrari, M., Garcia-Larsen, V., Jalil, A., Degan, P., Fois, A. G., Zinellu, E., Fois, S.S., Fratta Pasini, A.M., Nicolis, M., Olivieri, M., Corsico, A., Bono, R., Pirina, P., Zanolin, M.E., 2020. Biomarkers of Oxidative Stress and Inflammation in Chronic Airway Diseases, 18;21(12): Int J. Mol. Sci. 4339. https://doi.org/10.3390/ijms21124339.
Chen, T., Yan, J., Li, Y., 2014. Genotoxicity of titanium dioxide nanoparticles. J. Food Drug Anal. 22, 95–104. https://doi.org/10.1016/j.jfda.2014.01.008.
Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., Bankoglu, E.E., Bonassi, S., Boutet-Robinet, E., Brunborg, G., Chao, C., Cooke, M.S., Costa, C., Costa, S., Dhawan, A., de Lapuente, J., Bo', C.D., Dubus, J., Dusinska, M., Duthie, S.J., Yamani, N.E., Engelward, B., Gaivão, I., Giovannelli, L., Godschalk, R., Guilherme, S., Gutzkow, K.B., Habas, K., Hernández, A., Herrero, O., Isidori, M., Jha, A.N., Knasmüller, S., Kooter, I.M., Koppen, G., Kruszewski, M., Ladeira, C., Laffon, B., Larramendy, M., Hégarat, L.L., Lewies, A., Lewinska, A., Liwszyc, G.E., de Cerain, A.L., Manjanatha, M., Marcos, R., Milić, M., de Andrade, V.M., Moretti, M., Muruzabal, D., Novak, M., Oliveira, R., Olsen, A.K., Owiti, N., Pacheco, M., Pandey, A.K., Pfuhler, S., Pourrut, B., Reisinger, K., Rojas, E., Rundén-Pran, E., Sanz-Serrano, J., Shaposhnikov, S., Sipinen, V., Smeets, K., Stopper, H., Teixeira, J.P., Valdiglesias, V., Valverde, M., van Acker, F., van Schooten, F.J., Vasquez, M., Wentzel, J.F., Wnuk, M., Wouters, A., Žegura, B., Zikmund, T., Langie, S.A.S., Azqueta, A., 2023. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat. Protoc. 18 (3), 929–989. https://doi.org/10.1038/s41596-022-00754-y.
Demir, E., Akca, H., Turna, F., Aksakal, S., Burgucu, D., Kaya, B., Tokgün, O., Vales, G., Creus, A., Marcos, R., 2015. Genotoxic and cell-transforming effects of titanium dioxide nanoparticles. Environ. Res. 136, 300–308. https://doi.org/10.1016/j.envres.2014.10.032.
Durante, M., Formenti, S.C., 2018. Radiation-induced chromosomal aberrations and immunotherapy: micronuclei, cytosolic DNA, and interferon-production pathway. Oncology 8, 192. https://doi.org/10.3389/fonc.2018.00192.
European Chemicals Agency, 2021. ECHA Guide on the Classification and Labelling of Titanium Dioxide. European Chemicals Agency: Helsinki,, Finland.
Fenech, M., 2000. The in vitro micronucleus technique, 20 Mutat. Res. 455 (1–2), 81–95. https://doi.org/10.1016/s0027-5107(00)00065-8.
Fenech, M., Holland, N., Kirsch-Volders, M., Knudsen, L.E., Wagner, K.H., Stopper, H., Knasmueller, S., Bolognesi, C., El-Zein, R., Bonassi, S., 2020. Micronuclei and disease - Report of HUMN project workshop at Rennes 2019 EEMGS conference, 503133 Mutat. Res Genet Toxicol. Environ. Mutagen 850–851. https://doi.org/10.1016/j.mrgentox.2020.503133.
Fenech, M., Knasmueller, S., Knudsen, L.E., Kirsch-Volders, M., Deo, P., Franzke, B., Stopper, H., Andreassi, M.G., Bolognesi, C., Dhillon, V.S., Laffon, B., Wagner, K.H., Bonassi, S., 2021. Micronuclei and Disease" special issue: Aims, scope, and synthesis of outcomes. Mutat. Res Rev. Mutat. Res 788, 108384. https://doi.org/10.1016/j.mrrev.2021.108384.
Gea, M., Bonetta, S., Iannarelli, L., Giovannozzi, A.M., Maurino, V., Bonetta, S., Hodoroaba, V.D., Armato, C., Rossi, A.M., Schilirò, T., 2019. Shape-engineered titanium dioxide nanoparticles (TiO2-NPs): cytotoxicity and genotoxicity in bronchial epithelial cells. Food Chem. Toxicol. 127, 89–100. https://doi.org/10.1016/j.fct.2019.02.043.
Glei, M., Habermann, N., Osswald, K., Seidel, C., Persin, C., Jahreis, G., Pool-Zobel, B.L., 2005. Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet Assay: a biomarker model, 203-17 Biomarkers 10. https://doi.org/10.1080/13547500500138963.
Heinrich, U., Fuhst, R., Rittinghausen, S., Creutzenberg, O., Bellmann, B., Koch, W., Levsen, K., 1995. Chronic inhalation exposure of wistar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. Inha. Toxicol. 7, 533–556.
IARC, 2010, Titanium dioxide. In: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans: Carbon Black, Titanium Dioxide, and Talc, Vol. 93. Lyon: IARC, pp. 194–276.
Jalilian, H., Neghab, M., Tatar, M., Taheri, S., 2018. Respiratory and dermal symptoms and raised serum concentrations of biomarkers of oxidative stress among pesticide retailers. Int J. Occup. Environ. Med 9, 194–204. https://doi.org/10.15171/ijoem.2018.1417.
Kargar-Shouroki, F., Dehghan Banadkuki, M.R., Jambarsang, S., Emami, A., 2022. The association between wood dust exposure and respiratory disorders and oxidative stress among furniture workers. Wien. Klin. Woche 134 (13–14), 529–537. https://doi.org/10.1007/s00508-022-02048-5.
Kaushik, R., Khaliq, F., Subramaneyaan, M., Ahmed, R.S., 2012. Pulmonary dysfunctions, oxidative stress and DNA damage in brick kiln workers. Hum. Exp. Toxicol. 31 (11), 1083–1091. https://doi.org/10.1177/0960327112450899.
Larsen, S.T., Jackson, P., Poulsen, S.S., Levin, M., Jensen, K.A., Wallin, H., Nielsen, G.D., Koponen, I.K., 2016. Airway irritation, inflammation, and toxicity in mice following inhalation of metal oxide nanoparticles, 62 Nanotoxicology 10 (9), 1254. https://doi.org/10.1080/17435390.2016.1202350.
Lee, K.P., Trochimowicz, H.J., Reinhardt, C.F., 1985. Pulmonary response of rats exposed to titanium dioxide (TiO2) by inhalation for two years. Toxicol. Appl. Pharm. 79, 179–192.
Leonardi, S., Poma, A.M.G., Colafarina, S., D'alosio, F., Scatigna, M., Zarivi, O., Mastrantonio, R., Tobia, L., Fabiani, L., 2020. Early genotoxic damage through micronucleus test in exfoliated buccal cells and occupational dust expoure in construction workers: a cross-sectional study in L'Aquila, Italy. Ecotoxicol. Environ. Saf. 203, 110989 https://doi.org/10.1016/j.ecoenv.2020.110989.
Li, Y., Yan, J., Ding, W., Chen, Y., Pack, L.M., Chen, T., 2017. Genotoxicity and gene expression analyses of liver and lung tissues of mice treated with titanium dioxide nanoparticles. Mutagenesis 32, 33–46. https://doi.org/10.1093/mutage/gew065.
Liao, F., Chen, L., Liu, Y., Zhao, D., Peng, W., Wang, W., Feng, S., 2019. The size-dependent genotoxic potentials of titanium dioxide nanoparticles to endothelial cells. Environ. Toxicol. 34 (11), 1199–1207. https://doi.org/10.1002/tox.22821.
Lindberg, H.K., Falck, G.C., Catalán, J., Koivisto, A.J., Suhonen, S., Järventaus, H., Rossi, E.M., Nykäsenoja, H., Peltonen, Y., Moreno, C., Alenius, H., Tuomi, T., Savolainen, K.M., Norppa, H., 2012. Genotoxicity of inhaled nanosized TiO(2) in mice.. *Mutat Res*. 14, 745 (1–2), 58–64. https://doi.org/10.1016/j.mrgentox.2011.10.011.
Ling, C., An, H., Li, L., Wang, J., Lu, T., Wang, H., Hu, Y., Song, G., Liu, S., 2021. Genotoxicity Evaluation of titanium dioxide nanoparticles in vitro: a systematic review of the literature and meta-analysis. Biol. Trace Elem. Res. 199, 2057–2076. https://doi.org/10.1007/s12011-020-02311-8.
McCauley, L.A., Lasarev, M., Muniz, J., Stewart, V.N., Kisby, G., 2008. Analysis of pesticide exposure and DNA damage in immigrant farmworkers, 237-46 J. Agromed. 13. https://doi.org/10.1080/10599240802473817.
McKelvey, K.J., Hudson, A.L., Back, M., Eade, T., Diakos, C.I., 2018. Radiation, inflammation and the immune response in cancer. Mamm. Genome 29, 843–865. https://doi.org/10.1007/s00335-018-9777-0.
Muhle, H., Bellmann, B., Creutzenberg, O., Dasenbrock, C., Ernst, H., Kilpper, R., MacKenzie, J.C., Morrow, P., Mohr, U., Takenaka, S., 1991. Pulmonary response to toner upon chronic inhalation exposure in rats. Fundam. Appl. Toxicol. 17, 280–299. https://doi.org/10.1016/0272-0590(91)90219-t.

National Institute for Occupational Safety and Health (NIOSH), 2011, NIOSH current intelligence bulletin 63: occupational exposure to titanium dioxide. ⟨http://www.cdc.gov/niosh/docs/2011–160/pdfs/2011–160.pdf⟩. [Accessed 6 March 2023].

Osswald, K., Mittas, A., Glei, M., Pool-Zobel, B.L., 2003. New revival o fan old biomarker: characterisation of buccal cells and determination of genetic damage in the isolated fraction of viable leucocytes. Mutat. Res. 544, 321–329. https://doi.org/10.1016/jmrrev.2003.06.008.

Park, E.J., Lee, G.H., Shim, H.W., Kim, J.H., Cho, M.H., Kim, D.W., 2014. Comparison of toxicity of different nanorod-type TiO2 polymorphs in vivo and in vitro. J. Appl. Toxicol. 34 (4), 357–366. https://doi.org/10.1002/jat.2932.

Patel, S., Patel, P., Bakshi, S.R., 2017. Titanium dioxide nanoparticles: an in vitro study of DNA binding, chromosome aberration assay, and comet assay. Cytotechnology 69, 245–263. https://doi.org/10.1007/s10616-016-0054-3.

Pedrino, M., Brassolatti, P., Maragno Fattori, A.C., Bianchi, J., de Almeida Rodolpho, J. M., de Godoy, K.F., Assis, M., Longo, E., Nogueira Zambone Pinto Rossi, K., Speglich, C., de Freitas Anibal, F., 2022. Analysis of cytotoxicity and genotoxicity in a short-term dependent manner induced by a new titanium dioxide nanoparticle in murine fibroblast cells. Toxicol. Mech. Methods 32 (3), 213–223. https://doi.org/10.1080/15376516.2021.1994075.

Pelclova, D., Barosova, H., Kukutschova, J., Zdimal, V., Navratil, T., Fenclova, Z., Vlckova, S., Schwarz, J., Zikova, N., Kacer, P., Komarc, M., Belacek, J., Zakharov, S., 2015. Raman microspectroscopy of exhaled breath condensate and urine in workers exposed to fine and nano TiO2 particles: a cross-sectional study, 14 J. Breath. Res 9 (3), 036008. https://doi.org/10.1088/1752-7155/9/3/036008.

Russo, C., Acito, M., Fatigoni, C., Villarini, M., Moretti, M., 2020. B-Comet Assay (Comet Assay on Buccal Cells) for the Evaluation of Primary DNA Damage in Human Biomonitoring Studies. Int. J. Environ. Res. Public Health 17, 9234. https://doi.org/10.3390/ijerph17249234.

Shadab, M., Agrawal, D.K., Aslam, M., Islam, N., Ahmad, Z., 2014. Occupational Health Hazards among Sewage Workers: Oxidative Stress and Deranged Lung Functions, 8 (4):BC11-2 J. Clin. Diagn. Res.. https://doi.org/10.7860/JCDR/2014/5925.4291.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res 175 (1), 184–191. https://doi.org/10.1016/0014-4827(88)90265-0.

Thomas, P., Fenech, M., 2011. Buccal micronucleus cytome assay. Methods Mol. Biol. 682, 235–248. https://doi.org/10.1007/978-1-60327-409-8_17.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen 35, 206–221. https://doi.org/10.1002/(sici)1098-2280(2000)35:3<206::aid-em8>3.0.co;2-j.

Ursini, C.L., Di Basilio, M., Ciervo, A., Fresegna, A.M., Maiello, R., Buresti, G., Campopiano, A., Angelosanto, F., Papacchini, M., Iavicoli, S., Cavallo, D., 2021. Biomonitoring of workers employed in a titanium dioxide production plant: Use of buccal micronucleus cytome assay as noninvasive biomarker to evaluate genotoxic and cytotoxic effects. Environ. Mol. Mutagen 62 (4), 242–251. https://doi.org/10.1002/em.22431.

Vignardi, C.P., Hasue, F.M., Sartório, P.V., Cardoso, C.M., Machado, A.S., Passos, M.J., Santos, T.C., Nucci, J.M., Hewer, T.L., Watanabe, I.S., Gomes, V., Phan, N.V., 2015. Genotoxicity, potential cytotoxicity and cell uptake of titanium dioxide nanoparticles in the marine fish Trachinotus carolinus (Linnaeus, 1766), 2015 Aquat. Toxicol. 158, 218–229. https://doi.org/10.1016/j.aquatox.2014.11.008.

Villarini, M., Levorato, S., Salvatori, T., Ceretti, E., Bonetta, S., Carducci, A., Grassi, T., Vannini, S., Donato, F., Bonetta, S., Verani, M., de Donno, A., Bonizzoni, S., Bonetti, A., Moretti, M., Gelatti, U., MAPEC LIFE Study Group, 2018. Buccal micronucleus cytome assay in primary school children. A descriptive analysis of the MAPEC_LIFE multicenter cohort study. Int. J. Hyg. Environ. Health 221, 883–892. https://doi.org/10.1016/j.ijheh.2018.05.014.

Wani, M.R., Shadab, G., 2020. Titanium dioxide nanoparticle genotoxicity: A review of recent in vivo and in vitro studies. Toxicol. Ind. Health 36 (7), 514–530. https://doi.org/10.1177/0748233720936835.