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TiO₂ genotoxicity: an update of the results published over the last six years.

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Abstract

TiO₂ particles are broadly used in daily products, including cosmetics for their UV-absorbing property, food for their white colouring property, water and air purification systems, self-cleaning surfaces and photoconversion electrical devices for their photocatalytic properties. The toxicity of TiO₂ nano- and microparticles has been studied for decades, and part of this investigation has been dedicated to the identification of their potential impact on DNA, i.e., their genotoxicity. This review summarizes data retrieved from their genotoxicity testing during the past 6 years, encompassing both *in vitro* and *in vivo* studies, mostly performed on lung and intestinal models. It shows that TiO₂ particles, both nano- and micro-sized, produce genotoxic damage to a variety of cell types, even at low, realistic doses.

Keywords: TiO₂, genotoxicity, toxicity, lung, intestine, gut

1. Introduction

Worldwide production of titanium dioxide (TiO₂) approximates 5.5 million metric tons [1]. These mineral particles, either micro- or nano-sized, are mainly used as white colouring agent in paints, plastics, papers and food, but also in cosmetics and pharmaceuticals (www.tdma.info). When used as pigment, the average diameter of TiO₂ particle approximates 150 nm and less than 50% of the particles have a diameter smaller than 100 nm. Therefore, such materials are not considered as nanomaterials according to the European Commission. In its recommendation for a definition, a nanomaterial is “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm” [2]. When used as a UV filter in cosmetics, TiO₂ is either micro- or nanosized. The aspect of the applied sunscreen is dependent on the size: if transparent, TiO₂ is nanosized, if white, TiO₂ is rather micro-sized. Another application of TiO₂ particles is their use as catalysts in self-cleaning and anti-fogging tiles, windows, textiles etc. and in semi-conductor and solar cell industries. For these applications, TiO₂ particles are mostly nano-sized in order to take advantage of the high specific area of nanoparticles (NPs) and thus their better catalytic properties than microparticles (MPs).

Due to this wide use of TiO₂, populations are exposed *via* either ingestion, inhalation, or when products containing TiO₂ enter into contact with the skin. The use of TiO₂ in cosmetics is the main source of exposure of the populations via skin contact. The use of TiO₂ as a food additive has been authorized in 1969 by US-FDA and JECFA and is designated as E171 in the European Union [3]. This is the main source of exposure to TiO₂ via ingestion. The acceptable daily intake (ADI) allocated by JECFA was ‘not limited except for good manufacturing practice’ and in their re-evaluation in 2016 the EFSA panel on Food Additives and Nutrient Sources added to Food (ANS) did not establish an ADI due to the lack of reliable data on reproductive toxicity [4]. Oral exposure to TiO₂ has been estimated to range between 0.2 and 1 mg Ti/kg b.w./day in US adults and between 1 and 3 mg /kg b.w./d in children [5]. Recently, the EFSA refined these exposure levels when re-evaluating the authorization of E171. Two exposure scenarios were employed, based on i) maximum levels provided to EFSA, which was defined as the “maximum level exposure assessment scenario” and ii) reported use levels as provided by industry and the Member States, which was defined as the “refined exposure assessment scenario”. In the refined exposure assessment scenario, exposure was estimated to range from 0.2 mg/kg b.w. per day for infants and the elderly to 5.5

mg/kg b.w. per day for children and 4 mg/kg b.w. per day for adults, at the mean. These estimations were calculated using the non-brand-loyal scenario, which was considered as the more appropriate and realistic scenario for risk characterisation [4]. The inhalation exposure route concerns workers in production plants, who may inhale TiO₂ micro- or nanoparticles that can be suspended in the air of production plants during transfer of TiO₂ powder from one container to another, or during unloading or maintenance operations on production sites. TiO₂ is a very inert material, which is considered as non-soluble. For this reason, it has long been used as a negative control in inhalation studies evaluating nanoparticle toxicity (for instance in [6]). However, early studies also reported the development of lung tumors after inhalation exposure, for instance in animals exposed for two years by inhalation [7]. For this route of exposure, in 2005 the United States National Institute for Occupational Safety and Health (NIOSH) proposed a recommended exposure limit (REL) for TiO₂ NPs at 0.3 mg/m³ [8]. For total TiO₂ dust, the American Conference of Governmental Industrial Hygienists (ACGIH) allocated a threshold limit value of 10 mg/m³ as a time weighted average for a normal 8 h workday and a 40 h workweek [9]. TiO₂ has been classified as possible carcinogenic to humans (group 2B) by inhalation by the International Agency for Research on Cancer (IARC). Because of its widespread use, classification by IARC has led to intensive research on its toxicity, which is well reflected in the large number of publications on TiO₂ toxicity published over the last two decades. This classification also raised the question of its genotoxicity, since DNA damage may result in somatic mutations in the initiation step of cancer progression, which may later lead to malignant transformation. Genotoxicity testing of TiO₂-NPs has been one of the topics of the nanogenotox European project (2008-2013). From this project and other projects funded in the FP6, FP7 and H2020 framework programmes, it is now well established that nanomaterials interfere with some toxicity assays, particularly genotoxicity assays as discussed elsewhere [10, 11]. Regarding TiO₂, particles are white, opaque, they both absorb and reflect light and show autofluorescence. Consequently, they might cause optical interference with some assays. Due to their low toxicity, they accumulate in large quantities in cells, hindering proper observation and analysis of some markers and endpoints. For instance, accumulation of large aggregates of TiO₂ in the cell cytoplasm might hinder proper observation of micronuclei or gamma-H2AX foci. Moreover, TiO₂ shows photocatalytic properties, which imply that it releases reactive oxygen species (ROS) when exposed to light –even laboratory light-. If genotoxicity assays are not performed in the dark, this might lead to spurious oxidation of DNA, e.g., in the comet assay where the nuclear envelope is lysed and the TiO₂ particles that are accumulated in the cytoplasm can come into

direct contact with the DNA, or when extracting DNA for measuring oxidized DNA bases by mass spectroscopy. Also in the comet assay, large agglomerates of TiO₂ in the cell cytoplasm could interfere with the migration of DNA during electrophoresis. All these examples show how cautious one must be when assessing TiO₂ genotoxicity.

Two excellent reviews reporting the latest results on TiO₂ genotoxicity testing have been published in 2013 [11, 12]. The aim of the present article is to present an update of the results on the genotoxicity of TiO₂ particles, via both inhalation and ingestion exposure routes, published since that date. Only experimental studies were analysed, not epidemiological studies. We chose to structure this review by first analyzing the articles reporting results from *in vivo* experiments published during this period, and then analyzing results from *in vitro* studies. In each of these two subparts, we first briefly summarize the most important ideas highlighted in these two previous reviews [11, 12]. In 2016, the EFSA published an evaluation study relative to TiO₂ used as food additive, part of it reporting the genotoxicity of this additive based on the literature published up to 2016, i.e., most of the time before 2013. Analysis of this evaluation report is also included in each of these two subparts. Then to follow up on these ideas, we report in each subpart the most significant results published between 2013 and today.

2. TiO₂ genotoxicity via inhalation or ingestion, *in vivo*

2.1. *Lessons from the literature up to 2013: TiO₂ genotoxicity mechanisms are both primary and secondary, and are most of the time indirect*

In the current nomenclature, primary genotoxicity occurs when the genotoxic event takes place in the cell where the nanoparticle has accumulated –or came into contact-, whereas secondary genotoxicity results from an event that takes place in a surrounding cell [13]. For example, during inflammation phagocytes are activated and they release some ROS, which can affect surrounding cells and damage their DNA. Both primary and secondary genotoxicity can be direct or indirect, i.e. the cellular target is DNA or other biomolecules, respectively [13]. The recent re-evaluation of TiO₂ as food additive by EFSA included an evaluation of its genotoxicity, based on 38 articles published from studies performed *in vitro* and 17 articles published from studies performed *in vivo*. Among the *in vivo* studies, 5 of them had been performed on animals exposed by inhalation and the remaining 12 had been performed on animals exposed by ingestion or oral gavage or intraperitoneal injection. The panel concluded

that TiO₂ particles, either micro- or nanosized, are unlikely to represent a genotoxic hazard *in vivo* [4]. Some of these *in vivo* studies showed shortcomings, which led the panel to conclude of their non-relevance for risk assessment. As reported in this evaluation by EFSA, in 1997, Driscoll and colleagues showed increased frequency of HPRT mutations in alveolar type II cells 15 months after intratracheal instillation to rats at 100 mg/kg bw, which is a high dose, but not at 10 mg/kg bw. This mutagenicity was correlated with persistent lung inflammation [14]. And indeed inflammation is considered as one of the key mechanisms by which TiO₂ NPs cause toxicity [12]. *In vivo*, inflammation may be the source of secondary genotoxicity. Rehn et al. observed increased levels of 8-oxo-dGuo in lungs of rats 90 days after a single intratracheal instillation of 0.15 to 1.2 mg/kg bw of two types of TiO₂-NPs, one pristine and one coated with silica [15]. This was concomitant with mild inflammation. In 2012, Saber et al. observed the presence of DNA damage, detected via the comet assay, in lung lining fluid cells of mice instilled 24 h before with 54 µg of rutile coated TiO₂ particles with 288 or 120 nm diameter. No inflammation was observed in animals exposed to these particles, while 12 nm anatase TiO₂-NPs induced inflammation but no damage to DNA [16]. This shows that inflammation is not always correlated with DNA damage caused by TiO₂-NPs. Using comet assay, Naya et al. reported no DNA damage in rats exposed via single or repeated, intermittent instillation at 1 or 5 mg/kg bw of TiO₂-NPs (sacrifice 3 or 24 h after instillation) or 0.2 or 1 mg/kg bw of TiO₂-NPs once a week for 5 weeks (sacrifice 3 h after the last instillation), respectively [17]. Using inhalation, Lindberg et al. showed no increase in the level of DNA damage using the comet assay on lung epithelial type II cells and Clara cells and in the frequency of micronuclei in peripheral PCE, in mice exposed to 0.8, 7.2 or 28.5 mg/m³ of TiO₂-NPs [18]. However, the highest concentration induced significant inflammation, showing again that inflammation and genotoxicity are sometimes, but not always, correlated. The mechanisms of TiO₂ genotoxicity have been detailed in the review by Magdolenova et al. [11]. They encompass interaction with nuclear proteins involved in replication, transcription, DNA repair, interaction with the mitotic spindle or its components, disturbance of cell cycle checkpoints, the presence of ROS or transition metal at NP surface, interference with mitochondrial components leading to accumulation of ROS combined with inhibition of antioxidant defence, ROS produced by surrounding inflammatory cells, disruption of the normal DNA damage response etc. This suggests that TiO₂-NP genotoxicity are both primary and secondary, i.e., linked or not with inflammation, and is most of the time indirect.

2.2. *TiO₂ exposure via inhalation, in vivo: genotoxicity and overload dose*

When it comes to nanoparticle toxicity assessment, an important question is whether the exposure dose is realistic or not, and in the case of inhalation if the dose produces a macrophage clearance overload or not. This question has been recently discussed by scientific experts of inhalation toxicology, relative to the carcinogenic potential of nanomaterials [19, 20]; it applies also to their genotoxic and mutagenic potential. Relier et al. published a study in which rats had been by intratracheal instillation, at the doses of 0.5, 2.5 or 10 mg/kg b.w. [21]. The authors show significant elevation of the number of gamma-H2AX foci - representative of double strand breaks to DNA-, of micronuclei -representative of chromosomal damage-, and a positive outcome in the comet assay -representative of DNA breaks and/or alkali-labile sites-. No induction of gene mutation is reported in this study. Noteworthy, the genotoxic damage was observed 35 days post-exposure, and was significant only in animals exposed at the highest doses (2.5 and 10 mg/kg b.w.), which were shown to be doses that overloaded macrophage clearance capacities. This study underlines a frequent issue in *in vivo* studies dealing with nanoparticle lung toxicity, which is the use of very high, unrealistic doses, i.e. irrelevant exposure scenarios. Finally, two studies published by the group of U. Vogel show that some (but not all) TiO₂-NPs cause damage to DNA, as assessed via the alkaline comet assay, at non overload doses [22, 23]. All the tested TiO₂-NPs caused lung inflammation [22, 23].

2.3. *TiO₂ exposure via ingestion, in vivo: genotoxicity or not?*

Regarding exposure via the oral route, in addition to the publication of the EFSA report that concluded in the absence of genotoxicity *via* oral ingestion, in 2014 Chen et al. showed that oral exposure to TiO₂-NPs induced significant increase in the number of bone marrow cells positive for γ -H2AX staining, but did not increase the frequency of these cells. In this study, rats have been exposed to 50 or 200 mg TiO₂-NPs/kg b.w./day for 30 days, using 75 nm-diameter anatase NPs [24]. Bettini et al. showed that E171, the form of TiO₂ used as food additive, which is 100-150 nm in diameter, induced the spontaneous development of aberrant crypt foci, i.e. initiated colon carcinogenesis [25]. In this study, rats had been orally dosed for 100 days at 200 μ g or 10 mg E171/kg b.w./day in the drinking water, which are low exposure doses. Moreover, in rats previously treated with 1,2-dimethylhydrazine (DMH), which is a

classical inducer of colorectal carcinogenesis [26], exposure to E171 increased the size of preneoplastic lesions in the colon of exposed animals, showing promotion of colorectal carcinogenesis [25]. This suggests that this additive might be genotoxic, but no genotoxicity was observed using the comet assay in cells isolated from Peyer's patches [25]. More recently, the same protocol was applied to rats, this time with E171 prepared in a food matrix [27]. It caused no perturbation of immune homeostasis, and no preneoplastic lesions. To explain this discrepancy, as suggested by Blevins et al., the physico-chemical characteristics of TiO₂ particles would greatly differ when suspended in water, compared to a powder. For instance, TiO₂ has been reported to agglomerate in water [28]. This could explain the conflicting results, although in our own experience the E171 used in the study by Bettini et al. (which is the same as in our studies [29-31]) forms very stable suspensions in water and do not agglomerate (unpublished data). Moreover, when in contact with food matrix TiO₂ would be coated by a corona, while it would not be the case when dispersed in water. It is well established that the corona governs the interaction of particles with their surrounding environment, including intestinal cells and the microbiota [32]. In the case of TiO₂-NPs it is likely that this corona would impair direct –and harmful- interaction of the crystalline surface of TiO₂ with the gut microenvironment. Moreover, the food matrix could contain some antioxidant and chemoprotective substances, such as vegetal substances, which could counteract the pro-oxidant impact of TiO₂, as already described in red meat-rich diets [33]. This suggests that the presence of a food matrix provides an effective protection against the harmful effects of this food additive.

3. TiO₂ genotoxicity *in vitro*, on models representatives of the lungs and the intestine

3.1. Mechanisms of TiO₂-NP genotoxicity, using acute exposure scenarios

In addition to these *in vivo* studies, a larger number of studies have focused on the genotoxic impact of TiO₂ particles on *in vitro* lung and intestinal models. The outcomes of most of these studies are summarized and discussed in three excellent reviews published in 2013 and 2018 [11, 12, 34], to which the authors suggest the readers to refer for more details. Briefly, these reviews report that TiO₂ micro and/or nanoparticles induce strand breaks and alkali-labile sites, as assessed using the comet assay, as well as oxidized DNA bases including 8-oxo-dGuo, which is the most frequently oxidized base and consequently a sensitive marker of DNA oxidation. Some studies also report increased number of γ -H2AX foci, which is a

protein involved in the repair of DNA double strand breaks, and therefore is a marker of double strand breaks that can be considered as a marker of oxidative damage to DNA. Finally some studies report increased frequency of sister chromatid exchange and of HPRT gene mutation (hypoxanthine-guanine-phosphoribosyl-transferase gene, which is used as a model gene to investigate mutation in mammalian cells). The mechanisms of DNA damage caused by TiO₂ particles include accumulation of reactive oxygen species (ROS) in exposed cells, which may result from damage to mitochondria leading to increased leaching of ROS or from disturbance of the cellular antioxidant defence systems [12], which eventually fail to maintain redox homeostasis. In addition, TiO₂ particles can interact with DNA or with mitotic spindles during mitosis, leading to chromosomal damage [35]. Recently TiO₂-NPs have been suggested to disturb the DNA damage response by interacting with cell phosphoproteins, some of them being involved in cell cycle regulation, DNA repair, at the centre of which is the protein TP53 [36]. Finally, as other nanoparticles do, TiO₂ particles can accumulate in the cell cytoplasm via endocytosis [37], leading to the formation of very large vesicles filled with particles at the vicinity of cell nucleus. These large vesicles sometimes deform the shape of cell nucleus, and consequently it has been suggested that they may cause mechanical damage to the DNA [38]. Note however that for each of these endpoints, studies showing the opposite effect have also been published. As commented by Magdolenova et al. and Charles et al. [11, 34], this can be due to different factors including the tested materials (e.g. different size, shape, crystalline structure, surface roughness, presence of impurities, etc.), different procedures for preparing particle dispersion (presence of surfactant or proteins or not, use and parameters of sonication etc.), different cell types and test systems. This shows the difficulty of comparing *in vitro* experiments assessing nanomaterial genotoxicity, because the variety of test systems is wide. This suggests that it is not possible to draw general conclusions linking physico-chemical characteristics of TiO₂ particles with their toxic impact, and that each individual TiO₂ particle should be considered as a singular entity. Accordingly, its toxic impact should be assessed independently. Despite significant harmonization effort for nanoparticle dispersion and exposure procedures [39], it is still difficult to reach reproducible outcomes in genotoxicity experiments.

Before 2013, TiO₂ had also been reported to impair the capacity of the cell to repair DNA damage [40]. From 2013 until now, two studies suggested that the DNA damage caused by TiO₂-NP exposure might be efficiently repaired. El Yamani et al. showed that a 110 nm anatase TiO₂-NP caused some primary DNA lesions after 3 h of exposure in TK6 and A549

cells, while after 24 h of exposure this damage was less intense in TK6 cells and non-significant in A549 cells [41]. Di Bucchianico et al. showed the presence of primary DNA lesions in BEAS-2B cells exposed for 3 h to the same TiO₂-NP as the one used by El Yamani et al., as well as 20-28 nm rutile TiO₂-NP. These lesions were not observed after 24 h of exposure [42]. These studies are in agreement with an older one that showed the same trend, with an increased number of DNA lesions between 4 h and 24 h of exposure to TiO₂-NPs, then a drastic decrease in the level of lesions after 48 h of exposure, which suggest their repair [40]. However, Jugan et al. also showed strong reduction of DNA repair capacities in those cells exposed to TiO₂-NPs [40], which might somehow contradict the hypothesis of efficient DNA repair. Another possible explanation would be that the cells having high levels of DNA damage at the earlier time-point are dead at the latest time-point, and have been discarded from the cell culture during the washing steps prior to the comet assay.

3.2. Novel study designs and technical developments from 2013 to 2020

In addition to studies assessing TiO₂ genotoxicity based on acute exposure scenarios, several novel study designs and outcomes have been published from 2013 to recently. On lung-derived cell lines (Table 1), two teams have used chronic (repeated) exposure schemes to assess TiO₂ nanoparticle genotoxic potential on lung cells. Vales et al. showed that repeated exposure of BEAS-2 cells for 3 weeks to 12 nm anatase TiO₂-NPs (at 1 or 10 µg/mL) did not damage DNA as assessed via comet assay, in both its classical alkaline and Fpg-modified versions. In this study, cells were continuously grown in TiO₂-containing cell culture medium, with medium change every 4 days and subculture once a week [43]. Armand et al. reported that repeated exposure of A549 to 21 nm anatase/rutile TiO₂-NPs for up to two months, at 1 to 50 µg/mL, caused primary DNA lesions in alkaline and Fpg-modified comet assay [44]. The amplitude of this damaging effect increased as exposure duration and concentration increased. Moreover, cells repeatedly exposed to these NPs for 2 months showed increased sensitivity towards the alkylating agent methane methylsulfonate, suggesting impaired DNA repair capacities in exposed cells [44]. In the same condition, proteomics analysis showed that exposure to TiO₂-NPs modulated the level of some proteins involved in the DNA damage response, suggesting that this pathway was affected after repeated exposure of the cells to TiO₂-NPs [45]. Discrepancies between the studies by Vales et al. and Armand et al. might be explained by the exposure doses, which are slightly higher in the study by Armand et al.. Still, Armand et al. observe significant, although mild, DNA damage after exposing the cells to 10

$\mu\text{g/mL}$ from 24 h to 1 month in the comet-Fpg assay, which is comparable to the 3 weeks of exposure at 10 $\mu\text{g/mL}$ that induces no DNA damage in the study by Vales et al.. Besides, while Vales et al. used BEAS-2B cells, Armand et al. used A549 cells, which have been criticized because they show abnormal response in condition of oxidative stress [46]. However, A549 and BEAS-2B cells have also been shown to respond similarly to acute exposure to TiO_2 , with comparable genotoxic responses and comparable impact of TiO_2 -NPs on DNA repair capacities [47]. Yet, their response to chronic exposure to TiO_2 -NPs may differ.

Regarding the methodological developments related to the assessment of nanoparticles genotoxicity, higher throughput techniques have emerged in this period. The two recent studies by El Yamani et al. and Di Bucchianico et al. show the successful implementation of high-throughput comet assay (8 gels and 12 gels) in the assessment of TiO_2 -NP-induced primary DNA lesions [41, 42]. Di Bucchianico et al. validate the high-throughput flow-cytometry micronucleus assay [42]. Such efforts to develop high-throughput methods for toxicity testing are necessary in the context of rapid and constant development of new nanomaterials for which safety must be ensured [48]. Finally, in this period two studies showed the mutagenic potential of TiO_2 -NPs on lung cells. Chen et al. and Jain et al. showed that 75 nm anatase and 12-25 nm anatase TiO_2 -NPs, respectively, increased the mutation frequency in HPRT gene locus in V79 cells [24, 49].

3.3. Expansion of the literature reporting TiO_2 genotoxicity on intestinal models

Before 2013, the literature relative to TiO_2 genotoxicity was mainly focused on lung models. On intestinal models, Gerloff et al. had shown that TiO_2 induced DNA damage using comet assay when samples had been processed under normal laboratory lighting, whereas no significant DNA damage had been detected when processed in the dark [50]. In this study, Caco-2 cells had been exposed to TiO_2 -NPs (anatase/rutile, 20-80 nm, similar to NM105, and fine anatase TiO_2 particles) less than 24 h after their seeding, in serum-free cell culture medium. TiO_2 -NP suspensions had been prepared in serum-free cell culture medium and sonicated using an ultrasonic bath. The same authors had shown that the DNA damaging potential of TiO_2 particles was dependent on their physicochemical characteristics, with mixed anatase/rutile crystal phase showing significant effect while pure anatase samples induced no significant DNA damage [51].

After 2013, the literature reporting genotoxicity of TiO₂-NPs on intestinal cell models (either NPs or the food additive E171) has expanded considerably (Table 2). In 2015, Zijno et al. exposed non-differentiated Caco-2 cells to anatase TiO₂-NPs (<25 nm according to the supplier), prepared as a suspension in serum-free cell culture medium and then sonicated using an ultrasonic bath [52], as in the studies by Gerloff et al. [50, 51]. These NPs produced ROS in acellular condition, as shown using electron paramagnetic resonance with nitron spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), which revealed the presence of hydroxyl radical ([•]OH). Neither a significant increase of binucleate cells (BN) containing micronuclei (MN) nor a significant reduction of the replication index was observed in the cytochalasin-block micronucleus assay (CBMN) after treatment with TiO₂-NPs up to 20 µg/cm² (128 µg/mL) for 6 h or 24 h. In the Fpg-modified comet assay, positive outcome was obtained after 2 h of exposure to 2.5 µg/cm² (16 µg/mL) TiO₂-NPs but not at later time points, suggesting efficient repair of these primary lesions to DNA or, as discussed in section 3.1, elimination of the most damaged cells. No damage was observed in the alkaline or the EndoIII modified versions of the assay. Fpg and EndoIII are endonucleases that recognizes oxidized purines (8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua) and 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines), and oxidized pyrimidines (thymine glycol and uracil glycol), respectively. Therefore, when using these enzymes in the comet assay, these lesions are revealed in addition to strand breaks and alkali-labile sites. Increased levels of 8-oxo-dGuo were measured after exposure for 6 h or 24 h to TiO₂-NPs, which were efficiently repaired. Increased hOGG1 expression was measured in exposed cells, which supports the hypothesis of enhanced DNA repair activities in cells exposed to TiO₂-NPs [52]. Dorier et al. examined the toxicity of anatase and rutile TiO₂-NPs with diameter 12 nm and 20 nm, respectively, in non-differentiated Caco-2 cells. These NPs had been prepared by ultrasonication in water, then diluted in complete cell culture medium. Upon exposure for 6 h, 24 h or 48 h these NPs did not induce any DNA strand breaks in the alkaline version of the comet assay [53]. In 2018, Vila et al. studied the genotoxicity of 104 nm anatase TiO₂-NPs, which were dispersed according to the standard operating protocol of the Nanogenotox project (dispersion in BSA, high energy sonication, dilution in serum-containing cell culture medium). These particles induced a mild positive outcome in the alkaline version of the comet assay but not in the Fpg-modified version, in differentiated Caco-2 cells. These primary lesions to DNA were observed only at the lower concentration, i.e., 10 µg/mL, after 24 h of exposure [54]. The same team assessed the genotoxicity of TiO₂ nanospheres, nanorods or nanowires on a co-culture of

differentiated Caco-2 and HT29 cells. Again, a positive outcome was obtained in the comet assay (alkaline, but not Fpg-modified) in cells exposed to these three NPs for 24 h, which was not always dependent on the concentration of NPs. After 48 h of exposure, only TiO₂ nanorods still showed significant genotoxicity. Noteworthy, TiO₂-NPs located inside cell nuclei, as shown via confocal fluorescence imaging [55], which would imply that they may come into direct contact with DNA and consequently cause direct primary genotoxicity. This result is striking because insoluble nanoparticles have rather been described as causing indirect genotoxicity via the generation of ROS [11, 13]. In this study the authors used a co-culture of Caco-2 with HT29 cells and not HT29-MTX cells, which produce a large amount of mucus, while HT29 cells produce much less mucus [56]. Mucus is a protective substance that would impair direct contact between NPs and intestinal epithelial cells, therefore the use of HT29 rather than HT29-MTX cells could explain why some significant damage to DNA is observed in the study by Vila et al., compared to other studies published in the same period. For instance, in 2019, Dorier et al. showed that neither the food additive E171, nor 12 nm anatase TiO₂-NPs nor 21 nm anatase/rutile TiO₂-NPs induced any DNA damage in a non-differentiated Caco-2/HT29-MTX co-culture. This was demonstrated via the alkaline and Fpg-modified versions of the comet assay, via 53BP1 immunostaining that probes the presence of double-strand breaks (similar to gamma-H2AX, 53BP1 is another enzyme involved in DNA double strand breaks repair) and via measurement of 8-oxo-dGuo levels in cells exposed to 50 µg/mL of NM105 or E171. Moreover, the mRNA expression of OGG1, APE1, PARP1, LIG3, XRCC1, PCNA and GADD45 was unchanged, suggesting no impact on DNA repair mechanisms [31]. In this study, particle suspensions had been prepared by dispersing them in ultrapure water and ultrasonicated, then dilution in cell culture medium containing serum. In opposition to these 5 studies showing mild or no DNA damaging effect of TiO₂ particles on Caco-2 or Caco-2/HT29 or HT29-MTX cells, in 2017 Proquin et al. showed significant genotoxicity of E171 as well as TiO₂ micro- and nanoparticles (MPs and NPs, respectively) in intestinal cells [57]. In their study, the particles were prepared by suspension in medium containing bovine serum albumin (BSA) or serum, then bath sonication. E171, MPs and NPs induced the same extent of DNA damage in the comet assay, and E171 induced significant and concentration-dependent increase of the number of MN in BN cells in the CBMN assay, at 5, 10 and 50 µg/cm² (corresponding to 50, 100 and 500 µg/mL), in HCT116 cells [57]. At these concentrations, E171 was not cytotoxic but was shown to interact with the centromere region of kinetochore poles during mitosis [57]. This different result might be explained by different dispersion and exposure procedures, in which

TiO₂-NPs may have different agglomeration status and surface properties, including different protein corona.

4. Concluding remarks

During the 2013-2019 period, the research on TiO₂ particle genotoxicity has still been intense. Significant efforts have been devoted to the harmonization of test materials and their dispersion procedure, as exemplified by the broad use of the NM series of TiO₂-NPs provided by the European Joint Research Center and by the standard operating procedure for nanomaterial dispersion developed in the Nanogenotox project and applied in most of the following EU-funded projects. This makes possible direct comparison of results obtained by different teams, which ensures the robustness of data. Almost all articles published during this period show good quality physico-chemical characterization of TiO₂-NPs, at least on initial particles. Characterization of these materials during the course of exposure is most of the time lacking, as well as assessment of the interference of the NP with the test system. Some studies still report genotoxicity assessment in models that have been exposed to unrealistically high concentrations of TiO₂-NPs, but most of the time absence of cytotoxicity is also reported at these concentrations, as recommended in the guidelines for genotoxicity assessment. This ensures that the genotoxic damage is not due to cytotoxicity. Regarding the exposure systems, research dedicated to genotoxicity assessment of TiO₂-NPs on intestinal models have expanded, and new strategies to assess TiO₂-NP genotoxicity using chronic (repeated) exposure scenarios have emerged. Research is ongoing to develop more advanced *in vitro* models, such as 2D co-cultures or 3D cultures of spheroids or organoids, air-liquid interface exposure of lung cells, which could be used in the future as alternatives to *in vivo* testing. Up to now, these systems have not been used for genotoxicity assessment of TiO₂-NPs. Overall, the area of TiO₂-NP genotoxicity assessment is still very active, even if research is still needed in order to draw definitive conclusions about their potential genotoxic impact. In particular, in most articles published during this period, genotoxicity is assessed via the alkaline and enzyme-modified comet assay; it is less often tested *via* the micronucleus assay, and rarely *via* a gene mutation assays. In 2018, the EFSA recommended particle genotoxicity to be assessed *via* a battery of tests following OECD guidance. *In vitro*, this battery of tests should include a gene mutation assay, an assay testing chromosomal aberration and possibly the comet assay that would provide additional information. *In vivo*, if any *in vitro* assay shows positive outcome, genotoxicity should be tested via the micronucleus assay, the alkaline

comet assay and/or a gene mutation assay. In this context, effort has been devoted recently to the development and validation of high throughput methods for nanoparticle genotoxicity assessment, in particular the micronucleus assay using flow cytometry or automated microscopy (high content analysis) and procedures to safely isolate *in vivo* cells for comet assay. Some gene mutation assays that can be implemented as high throughput assays are also being developed, such as the PIG-A assay.

In summary, both *in vitro* and *in vivo* studies published during the period show that TiO₂ induce DNA damage, even at doses that do not overload macrophage clearance capacity. However, despite intense research effort coupled to the significant effort to harmonize protocols; despite the use of standard well-characterized model TiO₂-NPs, studies showing conflicting results have also been published. The use of more realistic models emerged and should be encouraged, as it would probably narrow the gap between *in vivo* and *in vitro* experimental protocols. Currently, secondary genotoxicity can only be assessed *in vivo*, but it would be possible to assess it in some advanced *in vitro* models such as co-cultures, organoids or organs-on-chips, which can recapitulate the complexity of organs and allow the dialogue between different cell types. Primary genotoxicity can be assessed both *in vivo* and *in vitro*. *In vitro* experiments can more easily provide mechanistic information, which is needed to appropriately understand the mode of genotoxic action of NPs. In these experiments, absence of interference of NPs with the assay has to be documented and the applied dose –or concentration- of NPs should be carefully chosen in order to ensure that saturation or overloading effects are avoided. The expert community in genotoxicity testing recommends the use of some specific cell lines for genotoxicity assessment, such as CHO-K1, V79 or TK6. Conversely, the community expert in nanoparticle toxicology recommends the use of human cell lines in order to assess appropriately the toxicity of NPs towards humans. The use of alternative cell lines to those recommended by genotoxicology experts is possible, but the sensitivity of each cell line should be documented, because not all cell lines show equivalent response to genotoxic agents. For instance, it would be valuable to compare the response of such cell line to a known genotoxic agent before using it for NP genotoxicity assessment, and in order to provide historical controls. Finally, the use of more realistic exposure scenarios, such as repeated exposure, exposure with a recovery period, air-liquid exposure has emerged, it would also more realistically recapitulate chronic or inhalation exposure of humans to NPs.

5. Declaration of interest

None

6. Acknowledgements

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1 Table 1. TiO₂ genotoxicity on lung models^a

Study	Cell line	Size	Crystalline structure	Dispersion procedure	Outcome
Chen et al., 2014 [24]	V79	75 nm from TEM	anatase	ultrasonication for 15 min (no more detail)	Positive outcome in alkaline comet assay at 100 µg/mL, 24 h (tested at 5, 20 and 100 µg/mL, 6 h and 24 h). Increased mutation frequency in the HPRT gene locus at 100 µg/mL, 24 h (tested at 5, 20 and 100 µg/mL, 24 h).
Kansara et al., 2015 [58]	A549	4-8 nm (TEM)		TiO ₂ -NPs purchased as a suspension, no other dispersion procedure	Positive outcome in alkaline comet assay and micronucleus assay at 75 and 100 µg/mL, 6 h (tested at 25, 50, 75 and 100 µg/mL, 6 h)
Vales et al., 2015 [43]	BEAS-2B	21 nm from TEM	anatase (NM102)	Probe sonication in 0.05% BSA, 16 min	Repeated exposure for 4 weeks (subculture weekly and medium change every 4 days) at 1, 10 and 20 µg/mL. Negative outcome in comet assay and MN assay, but increased number of clones growing in an anchorage-dependent colony formation assay (soft agar assay). Suggests a potential carcinogenic risk not mediated by a genotoxicity mechanism.
Armand et al., 2016 [44]	A549	22 nm from TEM	anatase/rutile (NM105)	Probe sonication in water, 30 min, 1s on/1s off	Positive outcome in comet assay, both alkaline and Fpg-modified at all tested concentrations (subculture two times a week, medium change every 3 or 4 days. tested at 5, 10, 50, 100 µg/mL up to 2 months). Sensitization to methyl methanesulfoxide of cells after repeated exposure to TiO ₂ -NPs, suggesting impaired DNA repair mechanisms.
Hanot et al., 2016 [59]	co-culture of THP-1 and HPMEC-ST1.6R microvascular cells (model of alveolo-capillar barrier), A549	22 nm according to JRC (not measured)	anatase/rutile (P25)	Indirect cup-type sonication (high energy) in water, 10 min, 1 min on/1 min off in RPMI+5% FBS+1% antibiotics	Phosphorylation of H2AX measured via Western blot in THP-1 and HPMEC cells, but not A549 at 200 and 800 µg/mL, 24 h (tested at 5, 200 and 800 µg/mL, 24 h). ????

Wallin et al., 2016 [23]	Mice, intratracheal instillation	both NRCWE-001 and -002: 10 nm according to the supplier	rutile (NRCWE-001 and NRCWE-002)	Indirect cup-type sonication (high energy) in water, 16 min	Positive outcome of alkaline comet assay (tail length) in lung at day 1 at all doses and both NPs (18, 54 and 162 µg/institution), at day 3, only at the lowest doses (NRCWE-002), at day 28, at all doses and both NPs. In bronchoalveolar lavage cells, positive outcome at day 3 at the lowest doses. In liver cells, positive outcome at day 1 and day 28 with NRCWE-001.
Hadrup et al., 2016 [22]	Mice, intratracheal instillation	NRCWE-001 and -002: 10 nm according to the supplier; NRCWE-025: 38 nm from AFM	rutile (NRCWE-001, NRCWE-002 and NRCWE-025)	Indirect cup-type sonication (high energy) in water or in 2% serum or in 0.05% BSA water/ethanol, 16 min	Positive outcome in alkaline comet assay in bronchoalveolar lavage cells, 24 h after exposure, for NRCWE-025 only, at 67 µg/institution (mice) when administered in water or in 2% serum or in 0.05% BSA water/ethanol
Biola-Clier et al., 2017 [47]	BEAS-2B and A549	22 nm according to JRC (not measured)	anatase/rutile (NM105)	Probe sonication in water, 30 min, 1s on/1s off	Positive outcome in alkaline comet assay and increased level of 8-oxo-dGuo at 100 µg/mL, 24 h (tested at 10 and 100 µg/mL), with more damage in A549 than in BEAS-2B cells (mild difference). DNA repair activity decreased in both cell types (NER pathway more severely affected than BER pathway). Decreased mRNA expression of some DNA repair proteins.
Di Bucchianico et al., 2017 [42]	BEAS-2B	50-100 nm (NM100), 5-8 nm (NM101), 20-28 nm (NM103) according to JRC (not measured)	anatase (NM100 and NM101) and rutile (NM103)	Probe sonication in 0.05% BSA, 16 min	Positive outcome in Fpg-modified comet assay for NM100 at 5 and 15 µg/mL and NM103 at 15 µg/mL (3 h) and for NM101 at 15 µg/mL (24 h) (tested at 1, 5, 15 µg/mL for 3 and 24 h, with both alkaline and Fpg-modified comet assays). Increased number of MN in binucleated cells at 1 and 5 µg/mL of NM103 and 1 µg/mL of NM101, 48 h (tested at 1, 5, 15, 30 µg/mL for 48 h)
El Yamani et al., 2017 [41]	A549 and TK6	110 nm from TEM	anatase (NM100)	Probe sonication in 0.05% BSA, 16 min	Positive outcome in Fpg-modified comet assay (when measured as strand breaks+Fpg) from 0.42 to 140 µg/mL (3h) and at 140 µg/mL (24 h) in TK6 cells (tested at 0.14, 0.42, 1.4, 4.2, 14, 42, 140 µg/mL for 3 h or 24 h). From 1 to 30 µg/cm ² (3 h) in A549 cells (tested at 0.1, 0.3, 1, 3, 10, 30 µg/cm ² for 3 h or 24 h). Dose-response.

Gosh et al., 2017 [60]	16HBE	21 nm (NM102), 26 nm (NM104), 21 nm (NM105)	anatase (NM102), rutile (NM104), anatase/rutile (NM105)	Probe sonication in 2% FBS, 16 min	Positive outcome in alkaline comet assay at 12.5 and 25 µg/mL (3h, NM102), 25 µg/mL (3h, NM104), 3.125, 12.5 and 25 µg/mL (24 h, NM102 and NM104), 12.5 and 25 µg/mL (24 h, NM105). No dose-response. No significant increase of MN (tested at 3.125, 12.5 and 25 µg/mL, 3 h or 24 h).
Jain et al., 2017 [49]	V79	12-25 nm from TEM	anatase	suspension in cell culture medium, no sonication	Increased number of HGPRT gene mutation at 50 and 100 µg/mL, 6 h (tested at 1, 10, 25, 50, 100 µg/mL, 6 h). Positive outcome in the alkaline comet assay at 25, 50 and 100 µg/mL, 6 h (tested at 1, 10, 25, 50, 100 µg/mL, 6 h). Dose-response. Approximately 20% cell death at these concentrations.
Stoccoro et al., 2017 [61]	A549	83 nm (A), 57 nm (A-Cit), 155 nm (A-SiO ₂), 489 nm (P25) from DLS	anatase and rutile: pristine (A), coated with citrate (A-Cit) or with silica (A-SiO ₂), P25	A, A-Cit and A-SiO ₂ provided as stable suspension. P25: bath sonication in water, then mixed with 0.05% BSA, then mixed with cell culture medium	Significant increase in MN frequency at all tested concentration and with all 4 NPs, except A-SiO ₂ at 10 µg/cm ² . Positive outcome in alkaline comet assay at 40 µg/cm ² (A), 20 and 40 µg/cm ² (A-Cit) and 10, 20 and 40 µg/cm ² (P25); in EndoIII-modified comet assay in all tested conditions; in Fpg-modified comet assay at 40 µg/cm ² of A, A-SiO ₂ and P25. (Both assays performed with exposure at 10, 20 and 40 µg/cm ² for 48 h)
Thongkam et al., 2017 [62]	A549	7 nm (NM101), 10 nm (NRCWE-001, -002, -003), 94 nm (NRCWE-004)	anatase (NM101), rutile (NRCWE-001, -002, -003, -004)	Procedure developed in the ENPRA EU-funded project	Positive outcome in the alkaline comet assay, but overall low level of DNA damage. Strongest effect with NM101.
Gea et al., 2019 [63]	BEAS-2B	50 nm (bipyramids), 108 nm (rods), 75 nm (platelets), 20 nm (P25), 150 nm (food-grade)	bipyramid, rods, platelets, P25, food-grade TiO ₂	Bath sonication, 100 min	Positive outcome in alkaline and Fpg-modified comet assay at all tested concentrations with food-grade and platelets; in Fpg-modified comet assay at all tested concentrations with P25 (tested at 80, 120 and 160 µg/mL, 24 h). Strongest effect with food-grade, then platelets, then P25.

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3 “Abbreviations: bovine serum albumin (BSA), foetal bovine serum (FBS), Joint Research Centre of the European Commission (JRC), micronucleus (MN), nanoparticle (NP),
4 transmission electron microscopy (TEM), Dynamic light scattering (DLS), X-ray diffraction (XRD).

5 Table 2. TiO₂ genotoxicity on intestinal models^a

Study	Cell line	Size	Crystalline structure	Dispersion procedure	Outcome
Chen et al., 2014 [24]	Rats, intragastric administration	75 nm from TEM	anatase	ultrasonication for 15 min (no more detail)	Increased number of gamma-H2AX foci in bone marrow cells at 50 and 200 mg/kg b.w./d, but no significant increase of MN frequency in bone marrow cells. (Daily exposure for 30 days at 0, 10, 50, 100, 200 mg/kg b.w./d)
Botelho et al., 2014 [64]	AGS gastric epithelial cells	421 nm from DLS measurement	anatase	Probe sonication for 5 min, 1.5 min on/1 min off in RPMI+10% FBS	Positive outcome in the alkaline comet assay at 150 µg/mL, 3 h.
Zijno et al., 2015 [52]	Caco-2, non differentiated	20-60 nm from TEM, SEM images	Anatase	Ultrasonic bath in FBS-free medium	No significant elevation of MN in binucleated cells (tested at 1, 2, 5, 10 µg/cm ² for 6 h or 24 h). Alkaline comet assay: positive only at 1 µg/cm ² for 4 h; Fpg-modified comet assay: positive only at 1 µg/cm ² , 1 h and 24 h (both tested at 1 and 2.5 µg/cm ² , 2 h, 4 h, 6 h, 24 h).
Dorier et al., 2015 [53]	Caco-2, non differentiated	12 nm (A-NP) and 22 nm (R-NP) from TEM images	Anatase (A-NP) and rutile (R-NP)	Probe sonication in water, 30 min, 1s on/1s off	Negative outcome in the alkaline comet assay (tested at 50 µg/mL for 6 h, 24 h, 48 h).
Donner et al., 2016 [65]	Rats, oral gavage	42 nm and 153 nm (anatase), 47, 195, 213 nm (rutile), 43 nm (anatase/rutile) from X-ray scanning disk centrifuge	2 samples of anatase, 3 samples of rutile, 1 sample of anatase/rutile	not indicated	no positive outcome in the MN assay (OECD TG 474) (tested concentrations: 500, 1000 and 2000 mg/kg b.w., single dose and red blood cells collected after 48 h or 72 h). No absorption from the gastrointestinal tract.
Bettini et al., 2017 [25]	Rats, oral gavage	22 nm (NP) and 20-340 nm (E171) from TEM images	Anatase/rutile (NP) and anatase (E171)	Probe sonication in 0.05% BSA, 16 min	Negative outcome in the alkaline comet assay in cells from Peyer's patches when exposed to E171, 10 mg/kg b.w./day, 7 days.
Proquin et al., 2017 [57]	Caco-2, non differentiated and HCT116	10-30 nm (NP), 250-1250 nm (MP), 50-250 (E171) from SEM images	Anatase (NP), MP and E171: not indicated	Ultrasonic bath 30 min, in DMEM+0.05% BSA (comet assay); McCoy 5A medium+10% SVF (MN)	Positive outcome in the comet assay at 0.143µg/cm ² for 24 h, with NP, MP and E171. Not concentration dependent (tested only with NP). Significant elevation of MN in binucleated cells at 5, 10 and 50 µg/cm ² for 24 h, concentration-dependent (E171; not tested with the other particles).

Dorier et al., 2017 [29]	Caco-2 and Caco-2/HT29-MTX, differentiated	12 nm and 24 nm (NP1 and 2), 118 nm (E171) from TEM images	Anatase (NP1 and E171), anatase/rutile (NP2)	Indirect sonication (high energy) in water, 30 min	cup-type (high energy) in water, 30 min	Positive outcome in Fpg-modified comet assay with E171 at 10 and 50 µg/mL, 24 h in Caco-2 cells and at 50 µg/mL, 24 h in Caco-2/HT29-MTX (all particle tested at 10 and 50 µg/mL, 24 h).
Vila et al., 2018 [54]	Caco-2, differentiated	104 nm from TEM images	Anatase (NM100 from JRC)	Probe sonication in 0.05% BSA, 16 min		Mild positive outcome at 10 µg/cm ² for 24 h in the alkaline comet assay only (tested at 10, 25 and 100 µg/cm ² for 24 h, via alkaline and Fpg-modified comet assay).
Garcia-Rodriguez et al., 2018 [55]	Caco-2/HT29, differentiated	30-160 nm (NP-sphere), D=20-180 nm and L=250 nm (NP-rod), D=4-26 nm and L=100 nm (NP-wire) from TEM images	Anatase (NP-sphere), rutile (NP-rod), and NP-wire	Probe sonication in 0.05% BSA, 16 min		Positive outcome in alkaline comet assay for the three NPs at 12.5, 50, 150, 350 µg/mL, i.e. all tested concentrations, 24h (except 350 µg/mL NP-wire); in Fpg-modified comet assay only with NP-rods (all tested concentrations) and NP-spheres (350 µg/mL only). Negative outcome in Fpg-modified comet assay (tested at 12.5, 50, 150, 350 µg/mL, 24 and 48 h).
Dorier et al., 2019 [31]	Caco-2/HT29-MTX, non differentiated	12 nm and 24 nm (NP1 and 2), 118 nm (E171) from TEM images	Pure anatase (NP1 and E171), anatase/rutile (NP2)	Indirect sonication (high energy) in water, 30 min	cup-type (high energy) in water, 30 min	Negative outcome in comet assay, alkaline and Fpg-modified (tested at 50 µg/mL for 24 h), in 53BP1 foci count assay (similar to gamma-H2AX). No significant increase of 8-oxo-dGuo level (HPLC-MS/MS).

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7 “Abbreviations: bovine serum albumin (BSA), foetal bovine serum (FBS), Hank’s balanced salt solution (HBSS), human serum albumin (HSA), Joint Research Centre of the
8 European Commission (JRC), micronucleus (MN), microparticle (MP), nanoparticle (NP), scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-
9 ray diffraction (XRD)

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