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Review Article

Toxicological Effects of Titanium Dioxide Nanoparticles: A Review of *In Vivo* Studies

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The essence of nanotechnology is the production of nanoparticles (NPs) with unique physicochemical properties allowing worldwide application in new structures, materials, and devices. The consequently increasing human exposure to NPs has raised concerns regarding their health and safety profiles. Titanium dioxide (TiO_2) has been reported to induce adverse pulmonary responses in exposed animals. However, the potential more dangerous biological activities of TiO_2 NPs compared to their fine-sized counterparts are not fully understood. Therefore, this work is aimed to provide a comprehensive evaluation of the toxic effects induced by TiO_2 NPs in *in vivo* experiments. It is intended to deeply understand the toxicological behaviour of TiO_2 NPs and to predict potential human health effects. Moreover, it may be an instrument to extrapolate relevant data for human risk evaluation and management and to identify those critical aspects that deserve great attention in future population and epidemiologic research.

1. Introduction

Nanotechnology is the manipulation of matter on a near-atomic scale to produce new structures, materials, and devises. It has became an important industry in the twenty-first century, and the U.S. National Science Foundation estimated it will grow into a trillion-dollar business, employing millions of workers worldwide, within the next decade [1–3].

The essence of nanotechnology is the synthesis of engineered nanoparticles (NPs) that exhibit characteristics, such as small size, large surface area to mass ratio, shape, crystallinity, surface charge, reactive surface groups, dissolution rate, state of agglomeration, or dispersal that confer them properties substantially different from those of the bulk particles of the same composition [4–6]. These properties offer great opportunities for the development of new NP industrial applications increasing their worldwide distribution and enhancing the likelihood of environmental and human exposure [5, 7].

Considerable work has been carried out to advance nanotechnology and its applications. Nevertheless, our understanding of the general and occupational health and safety aspects of NPs is still in its formative phase and greater effort is needed to understand how NPs interact with the human body [8, 9]. In this regard, nanotoxicology and nano-risk have been attracting the increasing attention of toxicologists and regulatory scientists [10] particularly in relation to the unique properties of NPs that may render them potentially more dangerous than their fine-sized counterpart and may cause unexpected adverse health effects to exposed people [6, 11].

Titanium dioxide (TiO_2) is an example of a fine, white, crystalline, odorless, low-solubility powder which was considered to exhibit relatively low toxicity [12–16]. It is a natural, thermally stable and nonflammable, nonsilicate mineral oxide found primarily in the form of the minerals rutile, anatase, brookite, and as the iron-containing mineral ilmenite [17–21]. It has excellent physicochemical properties, such as good fatigue strength, resistance to corrosion, machineability, biocompatibility, whitening and photocatalysis, as well as excellent optical performance and electrical properties [22, 23]. With regard to its potential adverse health effects, several studies have defined TiO_2, at least under nonoverload conditions, as biologically inactive and physiologically inert in both humans and animals and thus as little risk to humans [24–27]. Pulmonary inflammation, fibrosis, epithelial hyperplasia, and tumorigenesis were reported in animals under conditions of substantial TiO_2

particle lung burden due to sufficiently high dose and/or duration of exposure [13, 15, 28, 29]. In 2006, the International Agency for Research on Cancer (IARC), classified and in 2010 reassessed TiO_2 as "possibly carcinogenic to humans" on the basis of the sufficient evidence of carcinogenicity in experimental animals and inadequate evidence in humans (Group 2B) [28, 29].

TiO_2 is a versatile compound that has broadly been used in nanoparticulate form [21]. According to the National Nanotechnology Initiative of America, nanosized TiO_2 particles are among those most widely manufactured on a global scale [30]. TiO_2 NPs are widely used in paints, printing ink, rubber, paper, cosmetics, sunscreens, car materials, cleaning air products, industrial photocatalytic processes, and decomposing organic matters in wastewater due to their unique physical, chemical, and biological properties (including the inherent advantages of physical stability, anticorrosion and nanoscale-enhanced photocatalysis) [31]. However, the toxicological profile of TiO_2 NPs is not completely understood and several concerns have emerged on the potential undesirable effects of the TiO_2 NP properties, in regard to the harmful interactions with biological systems and the environment [11]. The recently recognized occupational carcinogenic potential of the inhaled TiO_2 NPs have ulteriourly enhanced these scientific concerns [15]. Therefore, an appropriate assessment of the risks for the general and occupational exposed population requiring TiO_2 NP hazard identification and dose-response data seems necessary [32].

In this regard, our previous work [33] reviewed several toxic effects of NPs assessed by *in vitro* experiments. These studies represent a valid instrument to investigate TiO_2 NP, induced cellular changes at biochemical and molecular levels and to determine their underlying mechanistic processes. Moreover, providing interesting information about doses of exposure and endpoint parameters to evaluate, this kind of investigation constitutes a significant step in planning animal research. Unfortunately, several limitations inherent to *in vitro* assay/cell culture systems have appeared in simulating the complex biological effects of particles administered to experimental animals. These include, but are not limited to, unrealistic particle dose, selection of cell types for simulating the different system microenvironments (single-cell culture systems or coculture systems), particle/cell exposure interactions in culture versus biological fluids, time course of effects represented by 1-, 4-, 24-, or 48-hour incubation *in vitro* versus acute or chronic exposure *in vivo*, and appropriate end points for hazard evaluation [34]. These biases do not allow to obtain data that may be representative of human exposure/effects and applicable for a correct population risk assessment.

Therefore, in the present review, we will provide a comprehensive evaluation of the current knowledge regarding the toxic effects induced by TiO_2 NPs on organ systems investigated in *in vivo* studies. This overview is intended to be a useful tool to gain a thorough insight into the toxicological profile of TiO_2 NPs and to predict potential human health effects. Moreover, it may reveal itself as a valid instrument to extrapolate relevant data for human risk evaluation and management and to identify those critical aspects that deserve great attention in future population and epidemiologic research.

2. *In Vivo* Studies

2.1. Respiratory System. Considering the relevance of the respiratory system as a route for TiO_2 NP exposure in humans, the scientific community has focused on the toxicological effects of these NPs on animal respiratory models. Such experiments provide the basis to obtain more detail data regarding the hazard of these NPs and to extrapolate evaluations for a correct human risk assessment, in particular, in relation to the characteristics of the particles. Indeed, several animal pulmonary studies were carried out to clarify the role played by TiO_2 form, particle size, shape, surface area, and chemistry in determining inflammatory responses, particle lung distribution, and carcinogenic effects (Table 1).

2.1.1. Inflammatory Responses

TiO_2 Form. Regarding the TiO_2 form, different studies have demonstrated the pulmonary toxicity of acute exposure to anatase in terms of increased bronchoalveolar lavage (BAL) inflammatory parameters [35–38], lung tissue structural damage, and inflammatory infiltration [22, 35, 36, 39, 40] although it is modest in both acute and subacute exposure [41]. However, as assessed by Liu et al. [36], only a slight increase in toxicity from anatase exposure was evident relative to rutile and P25 Degussa TiO_2 NP treatments.

Only four studies have evaluated the ability of rutile TiO_2 to induce toxic effects on lung tissues, reporting conflicting results following acute [42–45] or subchronic exposure [46]. While the first two studies demonstrated an increase of inflammatory cells in BAL [43], alterations in gene expression [42], and pathological changes in lung tissue [42, 43], the latter two failed to reveal such changes [44–46].

When comparing the lung toxicity induced by rutile NPs with that caused by P25 Degussa NPs, the former were not able to induce alterations of the lung parameters examined suggesting greater toxic effects of the mixture compared to rutile alone [44, 45]. Regarding the toxic effects of P25 Degussa NPs on the lung, other studies have reported that these particles are able to induce increased inflammatory parameters in the BAL of acutely exposed animals [47, 48], though some interspecies differences in inflammatory responses were evident in animals subchronically treated [49]. The species differences detected between hamsters, rats, and mice may reflect the capacity of some animals to rapidly clear particles from the lung, as previously demonstrated in the same species subchronically exposed to pigmentary fine TiO_2 particles [50, 51]. Interestingly, Gustafsson et al. [48] demonstrated a dynamic inflammatory response to P25 Degussa NPs characterized by a transient innate immune activation followed by a late-phase, long-lasting recruitment of lymphocytes involved in adaptive immunity.

Table 1: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on respiratory system.

		Respiratory system		
References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
Takenaka et al., 1986 [52]	Anatase TiO_2 (15–40)	**Inhalation:** aerosols of 8.6 mg/m^3 TiO_2 for 7 hr/day, 5 day/wk for up to 1 year.	24 female Wistar rats	**Lung burden:** TiO_2 was found in macrophages and in the perivascular spaces.
Ferin et al., 1992 [53]	Rutile TiO_2 (12; 230) Anatase TiO_2 (21; 250)	**Intratracheal instillation**: all TiO_2: 500 μg; 21 nm TiO_2: 65–1000 μg/rat; 250 nm TiO_2: 1000 μg/rat. **Inhalation:** 23.5 ± 3.2 mg/m^3 21 nm TiO_2; 23.0 ± 4.1 mg/m^3 250 nm TiO_2 for 6 h/day, 5 day/wk for up to 12 wks.	**Instillation study**: 4–8 male Fischer 344 rats **Inhalation study:** 4 male Fischer 344 rats per group.	**Instillation study**: unlavagable lung burden decreased from 12 nm to 250 nm particles. **Inhalation study**: greater 21 nm TiO_2 NP concentration in the interstitium, epithelial cells, and alveolar space.
Oberdorster et al., 1992 [54]	Anatase TiO_2 (~20; ~250) Rutile TiO_2 (~12; ~220)	**Basic intratracheal instillation:** 500 μg of 250 and 20 nm TiO_2. **Intratracheal reinstillation:** 104 ± 8 μg 20 nm free TiO_2, 104 ± 10 μg, 20 nm TiO_2 phagocitized by AMs, 100 μg serum coated particles, 6.8 × 10^6 AMs, 2.2 × 10^6 PMNs. **Intratracheal dose-response:** 500, 1000 μg 250 nm TiO_2; 65–1000 μg 20 nm TiO_2; 500 μg 220 nm TiO_2; 500 μg 12 nm TiO_2.	**Basic instillation:** 4 male Fischer 344 rats per group. **Reinstillation:** 4–10 male Fischer 344 rats per group.	**Basic instillation:** higher lung retained amount and greater inflammatory response in the 20 nm TiO_2 NP treatment. **Reinstillation:** greater amount of free particles retained in lavaged lungs. **Dose-response:** 20 nm TiO_2 NPs caused a dose-dependent PMN lung influx.
Oberdorster et al., 1994 [55]	Anatase TiO_2 (20; 250)	**Inhalation:** aerosols of 23.5 ± 2.9 mg/m^3 20 nm TiO_2 and 22.3 ± 4.2 mg/m^3 250 nm TiO_2 for 6 hr/day, 5 day/wk for up to 12 wks.	64 male Fischer 344 rats	**Inflammatory action:** AMs, PMNs, TPC increased in BAL. **Pulmonary retention**: increased after 20 nm TiO_2. **Histology:** lung fibrosis after 20 nm TiO_2.
Heinreich et al., 1995 [56]	P25 Degussa TiO_2 (15–40)	**Inhalation:** 10 mg/m^3 TiO_2 for 18 hr a day for 24 (rats) and 13.5 (mice) months.	100 female Wistar rats 80 NMRI mice	**Rats:** adenocarcinomas, squamous cell carcinomas, adenomas increased. **Mice:** no differences in tumor rates.
Baggs et al., 1997 [57]	Pigment-grade TiO_2 (250) Degussa TiO_2 (20)	**Inhalation:** aerosols of 22.3 mg/m^3 of 250 nm TiO_2 and 23.5 mg/m^3 of 20 nm TiO_2 for 6 hr/day, 5 day/wk for up to 12 wks.	3-4 male Fischer 344 rats per group	**Histology:** alveolar epithelial thickness and septal fibrosis after both TiO_2 treatments.
Zhang et al., 1998 [58]	TiO_2 (28)	**Intratracheal instillation**: 1 mg TiO_2.	4–6 male Wistar rats per group	**Inflammatory action:** TCC, NEUs, AMs, TPC, and LDH increased in BAL.
Afaq et al., 1998 [59]	TiO_2 (30)	**Intratracheal instillation**: 2 mg TiO_2 per animal.	6 female Albino rats per group	**Inflammatory action:** AMs, ACP, and LDH increased in BAL. **Oxidative stress:** LPO and antioxidant activities increased.

Table 1: Continued.

References	Crystal phase composition (particle size in nm)	Respiratory system Type of exposure	Type and number of animals	Results
Oberdorster et al., 2000 [60]	TiO_2 (20; 250)	**Intratracheal instillation**: no more detailed	Rats Mice	**Inflammatory action:** NEUs increased after 20 nm TiO_2.
Höhr et al., 2002 [61]	Native or methylated TiO_2 (20–30; 180)	**Intratracheal instillation**: equivalent mass (1 or 6 mg) and surface doses (100, 500, 600 and 3000 cm^2) of TiO_2 per animal.	5 female Wistar rats per group	**Inflammatory action:** native TiO_2 NPs increased TCC, PMNs, TPC, ALP, LDH, GGT, NAG and β glucuronidase in BAL.
Bermudez et al., 2004 [49]	P25 Degussa TiO_2 (21)	**Inhalation:** aerosols of 0.5–10 mg/m^3 TiO_2 for 6 hr/day, 5 days/week, for 13 weeks.	Groups of 25 animals for each species: Female 3C3F1/CrlBR mice; CDF (F344)/CrlBR rats; Lak:LVG (SYR) BR hamsters.	**Lung burdens:** equivalent in rats and mice, lower in hamsters. **Inflammatory action:** increase in TCC, AMs, NEUs, LYMs (rats and mice); TPC and LDH in BAL (rats); only NEUs in hamsters; **Histology:** terminal bronchiolar and alveolar cell replication.
Renwick et al., 2004 [62]	TiO_2 (29; 250)	**Intratracheal instillation**: 125, 500 μg TiO_2.	Male Wistar rats	**Inflammatory action:** NEUs, GGT, TPC, LDH increased after 29 nm TiO_2 in BAL. **AM phagocytotic ability**: decreased.
Warheit et al., 2006 [39]	Rutile pigment grade TiO_2 (300) Anatase TiO_2 rods (length: 92–233; wide: 20–35) Anatase TiO_2 dots (5.8–6.1)	**Intratracheal instillation**: 1, 5 mg/kg TiO_2.	5 male Crl:CD(SD)IGS BR rats per group	**Inflammatory action:** increase in NEUs (all particles) and LDH (TiO_2 rods). **Histology:** TiO_2-containing aggregated macrophages in lung (all particles).
Warheit et al., 2007 [44, 45]	~98% rutile TiO_2, 2% alumina (~100) ~88 wt% rutile TiO_2, ~7 wt% amorphous silica, ~5 wt% alumina (~100) P25 Degussa TiO_2 (~25) ~99% rutile TiO_2, ~1% alumina (382 in water)	**Intratrachel instillation:** 1, 5 mg/kg TiO_2.	4-5 male Crl:CD(SD)IGS BR rats per group	**Inflammatory action:** NEUs, LDH, and microprotein increased in BAL by P25 TiO_2 **Cell proliferation:** airway and lung parenchymal cells proliferation increased after P25 TiO_2. **Histology:** AM accumulation with tissue thickening after P25 TiO_2.
Chen et al., 2006 [42]	Rutile TiO_2 (21) TiO_2 (180–250)	**Intratracheal instillation**: 0.1, 0.5 mg TiO_2.	6 male ICR mice per group	**Lung morphology:** emphysematous change and alveolar epithelial thickness after 21 nm TiO_2 NPs. **Apoptosis:** increased in AMs and PNEs.
Grassian et al., 2007 [41]	Anatase TiO_2 (3.5 ± 1.0)	**Inhalation:** aerosols of 0.77, 7.22 mg/m^3 TiO_2 for 4 hr (acute exposure); and 8.88 ± 1.98 mg/m^3 TiO_2 for 4 hr/day for 10 days (subacute exposure).	6 male C57Bl/6 mice per group	**Acute exposure:** TCC and AMs increased in BAL. **Subacute exposure:** AMs increased in BAL.
Li et al., 2007 [35]	Anatase TiO_2 (3) TiO_2 (20)	**Intratracheal instillation**: 0.4–40 mg/kg TiO_2.	5 Male Kunming mice per group	**BAL biochemical parameters:** TPC, ALB, ALP, and ACP increased by both TiO_2. **Histology:** alveolar wall thickening and destruction.

Table 1: Continued.

References	Crystal phase composition (particle size in nm)	Respiratory system Type of exposure	Type and number of animals	Results
Geiser et al., 2005 [63]	TiO_2 (4)	**Inhalation:** 10 rats were exposed to 0.11 mg/m^3 TiO_2 aerosols for 1 hr.	10 male WKY/NCrl BR rats	**TiO_2distribution:** luminal side of airways/alveoli (79.3%), epithelial or endothelial cells (4.6%), connective tissue (4.8%), capillaries (11.3%).
Mühlfeld et al., 2007 [64]		See Geiser et al., 2005 [63]		**TiO_2distribution:** connective tissue and the capillary lumen were the preferential target of NPs at 1 and 24 hr, respectively.
Nemmar et al., 2008 [43]	Rutile TiO_2 nanorods (4–6)	**Intratracheal instillation**: 1, 5 mg/kg TiO_2	6-7 male Wistar rats per group	**Inflammatory action:** AMs, PMNs increased in BAL. **Tissue injury:** edema in lung and heart.
Larsen et al., 2010 [65]	TiO_2 (28)	**Intraperitoneal injections**: immunization with 1 μg OVA alone or in combination with either 2, 10, 50 or 250 μg TiO_2.	7-8 inbred female BALB/cJ mice per group	**OVA-specific antibodies in serum:** TiO_2 increased IgE and IgG1 levels compared to the OVA-controls. **Inflammatory action:** EOSs, NEUs, LYMs, IL-4, and IL-5 increased in BAL.
Hamilton et al., 2009 [37]	Anatase TiO_2 nanospheres (60 ~ 200) Anatase TiO_2 long nanobelts (width: 60 ~ 300 nm; length 15 ~ 30 nm) Anatase TiO_2 short nanobelts (width: 60 ~ 300; length 0.8–4 μm)	**Pharyngeal aspiration**: 30 μg/mouse TiO_2.	Male C57BL/6 mice	**Inflammatory action:** cathepsins, IL-1β, IL-18 increased after long nanobelts aspiration.
Kobayashi et al., 2009 [38]	Anatase TiO_2 (4.9) (1st and 2nd experiment) Anatase TiO_2 (23.4) Anatase TiO_2 (154.2)	**Intratracheal instillation**: 5 mg/kg TiO_2	5 male Crl: CD (SD) rats per group	**Inflammatory action:** **1st:** 4.9 and 23.4 nm TiO_2 NPs increased TCC, NEUs, LDH; **2nd:** agglomerated TiO_2 increased TCC, NEUs, LDH. **Histology:** epithelium hypertrophy in all treated groups.
Van Ravenzwaay et al., 2009 [66]	Anatase-rutile TiO_2 mixture (20–30) Rutile TiO_2 (200)	**Inhalation**: aerosols of 100 and 250 mg/m^3 uncoated and pigmentary TiO_2, respectively for 6 h/day on 5 consecutive days. **Intravenous injections**: 5 mg/kg.	3–6 male Wistar rats (strain Crl:WI (Han) per group	**TiO_2 distribution:** lung and mediastinal lymph node after inhalation; mostly liver and spleen after injection. **Inflammatory action:** both TiO_2 increased TCC, PMNs, TPC, ALP, LDH, GGT, NAG in BAL.
Ma-Hock et al., 2009 [67]	Rutile-anatase TiO_2 mixture (25.1)	**Inhalation**: aerosols of 2–50 mg/m^3 TiO_2 for 6 hr/day for 5 days.	5-6 male Wistar rats (strain Crl: WI (Han)) per group	**Inflammatory action:** PMNs, GGT, TPC, LDH, ALP, NAG increased in BAL. **Cell replication:** increased in bronchi and bronchioles.

Table 1: Continued.

References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
		Respiratory system		
Liu et al., 2009 [36]	Anatase TiO_2 (5) P25 Degussa TiO_2 (21) Rutile TiO_2 (50)	**Intratracheal instillation**: 0.5–50 mg/kg.	12 male and female Sprague Dawley rats per group	**Inflammatory action:** LDH and ALP increased in lung by 5 and 50 nm TiO_2. **Histology:** inflammatory infiltration, alveolar wall thickening. **AM phagocytic ability:** altered by 5 and 50 nm TiO_2.
Sager et al., 2008 [12]	P25 TiO_2 (21) Rutile TiO_2 (1 μm)	**Intratracheal instillation**: 0.26–1.04 mg/rat for 21 nm TiO_2 and 5.35–21.41 mg/rat for 1 μm TiO_2.	Male Fischer CDF (F344/DuCrl) rats	**Inflammatory action:** both TiO_2 increased PMNs, LDH, ALB, TNFα, IL-1β, MIP2. **Oxidative stress:** increased by both TiO_2.
Sager and Castranova, 2009 [68]	P25 TiO_2 (21)	**Intratracheal instillation**: 0.26–1.04 mg/rat TiO_2.	8 male Fischer CDF (F344/DuCrl) rats per group	**Inflammatory action:** increase in PMNs, ALB, and LDH in BAL.
Park et al., 2009 [69]	P25 TiO_2 (21)	**Intratracheal instillation**: 5, 20, 50 mg/kg TiO_2.	10–12 ICR mice per group	**Inflammatory action**: IL-1, TNF-α, IL-6, IL-12, IFN-γ, IL4, IL-5, IL-10, and IgE increased in BAL. **Histology:** inflammatory proteins, granulomas. **Gene expression:** upregulation of genes involved in antigen presentation and immune cell chemotaxis.
Chen et al., 2009 [22]	Anatase TiO_2 (80–110)	**Intraperitoneal injections**: 324–2592 mg/kg TiO_2.	10 male and female ICR mice per group	**Histology**: alveolar septal thickening and interstitial pneumonia.
Moon et al., 2010 [47]	P25 TiO_2 (21)	**Intraperitoneal injections**: 40 mg/kg TiO_2 alone or 30 min after 5 mg/kg LPS injection.	10 BALB/c mice per group	**Inflammatory action:** NEUs, TPC; TNF-α, IL-1β, MIP2 increased by TiO_2 or TiO_2+ LPS.
Rossi et al., 2010 [70]	Rutile TiO_2 (<5 μm) Rutile-anatase TiO_2 mixture (30–40) Anatase TiO_2 (<25) Silica coated rutile TiO_2 ($\sim 10 \times 40$) Anatase TiO_2/brookite (21)	**Inhalation:** 10 ± 2 mg/m^3 TiO_2 for 2 hr; 2 hr on 4 consecutive days; 2 hr on 4 consecutive days for 4 wks.	8 female BALB/c/Sca mice per group	**Inflammatory action:** NEUs in BAL, CXCL1 lung tissue, TNF-α in AMs increased by silica TiO_2.
Rossi et al., 2010 [71]	Silica coated rutile TiO_2 ($\sim 10 \times 40$) Rutile TiO_2 (<5 μm)	**Inhalation**: 10 ± 2 mg/m^3 TiO_2 for 2 hr a day, 3 days a wk, for 4 wks.	8 female BALB/c/Sca mice per group	**Inflammatory action:** EOSs, LYMs, AMs, PAS+ globet cells, IL-1β, TNF-α, IL-4, -13, -10 decreased by silica TiO_2. **Airway reactivity:** decreased by silica TiO_2; increased by fine TiO_2
Scuri et al., 2010 [72]	P25 Degussa TiO_2 (21)	**Inhalation**: 12 mg/m^3 TiO_2 for 5.6 hr a day, for 3 consecutive days.	29 male and female Fischer 344 rats	**Neurotrophin expression:** NGF, BDNF and their receptors increased in 2 day and 2 wk old rats. **Airway resistance:** increased in 2 wk old mice.
Li et al., 2010 [73]	Anatase TiO_2 (3)	**Intratracheal instillation:** 3.3 mg/kg TiO_2 once a wk for 4 wks.	13 male Kunming mice per group	**Inflammatory action:** ACP, ALP increased in BAL. **Histology:** destroyed alveolar walls.

Table 1: Continued.

References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
		Respiratory system		
Liu et al., 2010 [74]	TiO_2 (5) TiO_2 (200)	**Intratracheal instillation:** 0.5–50 mg/kg TiO_2	3 male and 3 female Sprague-Dawley rats per group	**AM phagocytic and chemotactic ability:** reduced by TiO_2 NPs.
Tang et al., 2010 [75]	TiO_2 (5)	**Intratracheal instillation:** 0.8–20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**TiO_2 NP aggregation:** present in lung at the lowest doses. **Histology:** lung gaps expanded, hyperemia
Tang et al., 2011 [40]	Anatase TiO_2 (5±1)	**Intratracheal instillation:** 0.8–20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**Histology:** AM increase, lung gaps expanded, hyperemia, alveolar thickness.
Cho et al., 2010 [32]	TiO_2 (30–40)	**Intratracheal instillation:** 50 and 150 cm^2/rat	5 female Wistar rats per group	**Inflammatory action in BAL and histology of the lung**: no effect.
Leppanen et al., 2011 [76]	Anatase + Brookite TiO_2 (20 nm)	**Inhalation:** 8–30 mg/m^3 for 0.5 hr (acute exposure); 30 mg/m^3 for 1 hr a day, 4 days a wk for 4 wks (sub-chronic exposure)	4–6 male Crl:OF1 mice per group	**Airflow limitation effect:** reduction in expiratory flow in all the exposure situations. **Inflammatory action:** no effect
Morimoto et al., 2011 [46]	Rutile TiO_2 (35)	**Inhalation**: $2.8 \pm 0.9 \times 10^5/cm^3$ 6 hr a day for 4 wks.	30 male Wistar rats per group	**Inflammatory action:** no effect.
Hougaard et al., 2010 [77]	Rutile UV-titan L181, modified with Zr, Si, Al and coated with polyalcohols (20.6 ± 0.3)	**Inhalation:** 42.4 ± 2.9 TiO_2 mg/m^3 for 1 hr a day, for 11 gestational consecutive days.	Female time-mated C57BL/6BomTac mice	**Inflammatory action:** increased NEUs, and LYMs, decreased AMs in BAL.
Halappanavar et al., 2011 [78]		See Hougaard et al., 2010 [77]		**Inflammatory action:** TiO_2 increased mRNA for Saa1, Saa3, several C-X-C and C-C motif chemokines, TNF-α.
Nemmar et al., 2011 [79]	Rutile Fe-doped nanorod TiO_2 (length: 80; diameter: 7)	**Intratracheal instillation:** 1,5 mg/kg TiO_2	4 male Wistar rats per group	**Inflammatory action:** NEUs, IL-6 increased, SOD activity decreased in BAL. **Histology:** inflammatory cell infiltration.
Hussain et al., 2011 [80]	Anatase TiO_2 (15)	**Oropharyngeal aspiration**: ~0.8 mg/kg TiO_2	5-6 male BALB/c mice	**Airway reactivity:** increased by TiO_2 in TDI sensitized mice **Inflammatory action:** TiO_2 increased NEUs and AMs in BAL of TDI sensitized mice.
Gustafsson et al., 2011 [48]	P25 Degussa TiO_2	**Intratracheal instillation:** 1, 5, and 7.5 mg/kg TiO_2	5–20 male Dark Agouti rats per group	**Inflammatory action**: transient increase in EOSs and NEUs in BAL, followed by a recruitment of DCs and NKs. Elevated levels of IL-1, IL-2, IL-6, CINC-1, and GM-CSF.

ACP, acid phosphatase; ALB, albumin; ALP, alkaline phosphatase; AM, alveolar macrophage; BAL, bronchoalveolar lavage; BDNF, brain-derived neurotrophic factor; CINC-1, cytokine induced neutrophil chemoattractant-1; CXCL-1, chemokine (C-X-C motif) ligand 1; DC, dendritic cell; NK, natural killer; EOS, eosinophil; GGT, γ-glutamyl transpeptidase; GM-CSF, granulocyte-macrophage colony-stimulating factor; Ig, immunoglobulin; IL-, interleukin; IFN-γ, interferon-γ; LDH, lactate dehydrogenase; LPO, lipid peroxidation; LPS, lipopolysaccharide; LYM, lymphocyte; MIP-2, macrophage-inflammatory protein-2; NAG, N-acetyl-glucosaminidase; NEU, neutrophil; NGF, nerve growth factor; PAS, Periodic Acid-Schiff; PMN, polymorphonuclear; PNE, pneumocyte; Saa, serum amyloid A-; SOD, superoxide dismutase; TiO_2 NPs, titanium dioxide nanoparticles; TCC, total cell count; TDI, toluene diisocyanate; TNF-α, tumor necrosis factor-α; TPC, total protein content.

Other mixtures of anatase and rutile particles were also able to induce increases in BAL fluid inflammatory parameters and lung histopathological alterations in rats subacutely exposed by inhalation [66, 67], while mixtures of anatase and brookite (3 : 1) failed to induce such effects in mice after acute, subacute, or subchronic inhalations [70, 76].

Other studies report similar changes after acute or subacute exposure, such as increased levels of inflammatory mediators in BAL [58, 59, 62, 73], enhanced reactive oxygen species (ROS) production [59] and pathological alterations at the histopathology examination of the lung [57, 73, 75] but lack details regarding the TiO_2 form. Interestingly, Baggs et al. [57] demonstrated that chronic exposure to TiO_2 NPs induces lesions that are able to regress during a 1-year period following cessation of exposure. Surprisingly, Cho et al. [32], investigating the acute effects of intratracheal instillations of TiO_2 NPs, failed to demonstrate any alteration among BAL inflammatory parameters and histological lung characteristics. Finally, Liu et al. [74] demonstrated that intratracheal instillation of not characterized TiO_2 NPs damaged alveolar macrophage (AM) phagocytic and chemotactic ability.

Size. Several studies described greater inflammatory effects of NPs compared to their fine counterparts both after acute [38, 53, 54, 62] and chronic exposure [53, 55].

In line with these results on NP size-related effects, a more recent intratracheal instillation study [38] pointed out that the smaller particles lead to greater inflammation in short-term observations. In contrast, no clear relationship was found between pulmonary inflammation and treatments with different agglomeration states of the same primary TiO_2 NPs.

Shape. The critical role of shape in TiO_2 NP bioactivity is supported by the increased markers of inflammation detected in BAL of anatase nanobelt aspiration-treated mice compared with those determined in animals treated with TiO_2 nanospheres [37].

Surface Area. High mass or volume dose of poorly soluble, low-toxicity (PSLT) fine particles in the lungs has been associated with overloading, while ultrafine particles impair lung clearance at lower mass or volume doses [15]. The increased lung retention and inflammatory response of nanosized PSLT particles compared to fine PSLT particles correlate better to the particle surface area dose [81, 82]. Indeed, the larger inflammatory response after TiO_2 NP treatment compared to larger particles may be ascribed to an increase in NP surface area. This conclusion has been questioned, and conflicting results are present in literature on this topic. Some studies support the hypothesis that surface area may be the more appropriate dose metric for TiO_2 NP pulmonary toxicity studies [12, 60, 61]. When the same mass doses were acutely introduced into rats and mice via intratracheal instillation, TiO_2 NPs induced a much greater pulmonary-inflammatory response than fine TiO_2 particles [12, 60]. However, when the doses were normalized to the particle-administered surface area, the response in the lung for both nanosized and fine TiO_2 particles showed the same dose-response curve. Thus, in pulmonary toxicity studies, surface area, for particles of different sizes but of the same chemistry, proved to be a better dosemetric parameter than particle mass or number.

However, other studies are in conflict with the notion that the inflammatory response is expected to be more severe with higher surface area NPs [39, 44, 45]. In fact, similar BAL cell count alterations were reported in rats after intratracheal administrations of the same dose of fine rutile TiO_2 particles, nanosized TiO_2 anatase rods and dots, although the latter had a >6-fold increase in surface area compared with the nanorods [39]. In a subsequent study, the same authors [44, 45] confirmed the previous evaluations and concluded that having a larger surface area does not necessarily indicate that NPs will produce greater pulmonary inflammation and cytotoxicity compared to larger particles of a similar composition. In line with the Warheit et al. [39, 44, 45] results, 2- to 5-nm anatase NPs (i.e., with the highest surface area and smallest particle size) were not particularly toxic in the Grassian et al. [41] subacute inhalation study. Furthermore, in Li et al. [35], 3-nm anatase NPs did not elicit more pulmonary toxicity compared to 20-nm NPs, irrespective of their smaller size and greater surface area.

However, an aspect to take in consideration for a correct interpretation of these data is the potential influence of "lung overload" [13]. In the last decade, it has become clear that a breakdown in normal AM-mediated clearance of particles is seen in overload [83]. This is thought to be a consequence of volumetric overload of the AM [84, 85] or a response to the greater particle surface area per mass dose, as in the case of NPs, which is associated with decreased AM clearance and inflammation [13, 86]. In this regard, the importance of the NP surface area overlaps with the role of the dose administered to rats. Most of the studies herein described showed greater TiO_2 NP effects at higher exposure doses [35, 36, 39, 40, 43–45, 48, 49, 61, 62, 65, 67, 79]. Unfortunately, the large dose range applied in these studies does not allow one to establish a direct relationship between the different treatments and pulmonary effects. Moreover, when a study employes unrealistically high doses of NPs, such as in the case of Chen et al. [22], its relevance is dubious at best. In this light, when analyzing the NP dose role in lung damage, caution should be applied in extrapolating data for human evaluations.

Chemistry. Warheit et al. [44, 45] found that only non-coated particles resulted in BAL alteration and lung histopathological changes compared to their coated counterparts, suggesting a possible influencing role of surface chemistry in nano-TiO_2-induced lung toxicity. In this regard, several studies have investigated the influence of particle chemical surface properties, surface coatings, and functionalization on pulmonary responses showing conflicting results [61, 70, 77, 78].

Methylated-hydrophobic TiO_2 NPs acutely decreased the percentage of PMN and the protein concentration in BAL of

intratracheally administered rats [61], a result in line with those of Oberdörster [87], but in contrast with those of Pott et al. [88] showing that hydrophobic, silanized TiO_2 NPs induced acute mortality in intratracheally exposed rats. Other studies reported significant inflammatory reactions after acute [70, 79] and subacute [70, 77, 78] exposure to coated NPs, particularly silica-coated rutile TiO_2 NPs [70], rutile TiO_2 NPs surface modified with unspecified amount of zirconium, silicon, aluminum, and coated with polyalcohols [77, 78] and rutile Fe-doped TiO_2 NPs [79]. However, at present, it is unclear what changes lead to differences in the toxicity induced by surface modified TiO_2 NPs. In additional experiments, P25 Degussa TiO_2 NPs caused more persistent pulmonary toxicity than carbon black NPs when compared on an equivalent particle surface area basis [68].

On the other hand, several studies investigated the TiO_2 NP role as an adjuvant in promoting allergic sensitization or in influencing allergic lung inflammation. In this regard, the results obtained by Larsen et al. [65] and Park et al. [69] supported the adjuvant effect of not characterized and P25 Degussa TiO_2 NPs, respectively, through the promotion of a T-helper (Th)-2 immune response assessed by increased levels of interleukin (IL)-4, IL-5, and IL-10 in BAL. These alterations could stimulate allergic reactions, though the underlying mechanisms are not fully understood. Interestingly, in two-day- and two-week-old rats, but not in adult animals, subacute inhalation of P25 Degussa TiO_2 NPs produced upregulation of lung neurotrophins which was associated with a greater airway hyperresponsiveness [72]. The age-dependent response to TiO_2 NPs suggests that the risk of developing asthma after NP exposure is higher in the earlier stages of lung development. Hussain et al. [80] demonstrated that intrapulmonary doses of TiO_2 NPs can aggravate pulmonary inflammation and airway hyperreactivity in a mouse model of diisocyanate-induced asthma. Moreover, mixture of anatase and brookite TiO_2 NPs caused airflow limitation in both acute and repeated 4-week-inhalation exposures [76]. In contrasts to these results [65, 69, 72, 76, 80], a recent study [71] demonstrated the role of silica-coated rutile TiO_2 NPs as inhibitors of most soluble and cellular mediators of allergic asthma. In fact, in ovalbumin-sensitized mice, the number of inflammatory cells, and the airway hyperreactivity were dramatically reduced after exposure to TiO_2 NPs. The conflicting results of the previous studies could be ascribed to the different health status of the animals, the different type of particle employed, or the different route of exposure. Rossi et al. [71] hypothesized that anti-inflammatory Th-2 response caused by allergen sensitization may be suppressed by the competing proinflammatory response elicited by TiO_2 exposure. However, future studies are needed to deeply clarify the TiO_2 NP role in allergic reactions and the molecular mechanisms involved.

2.1.2. Lung Distribution. Particle size was able to affect the fate of NPs, particularly lung accumulation and distribution in lung compartments, after pulmonary exposure. In fact, several studies described greater NP lung amounts compared to their fine counterparts [12, 52–55, 60]. Ferin et al. [53] suggested that TiO_2 particle passage in the interstitium could be promoted by the smaller sizes of the particles and the higher concentrations. Though data regarding the accumulation of NPs in the interstitium are too limited to allow a comprehensive evaluation of its consequences, this aspect is of particular interest considering that particle retention in the lung may give rise to potential enhancement of local effects and the increased possibility of systemic redistribution.

Translocation of NPs from pulmonary airways into other pulmonary compartments, such as epithelium/endothelium, connective tissue, the capillary lumen, or into systemic circulation, is the subject of controversial discussion in the literature. Two studies of the same group demonstrated conflicting results in that regard [63, 64]. The first showed that NP distribution was only related to the volume fractions of the corresponding compartments [63], while the second detected a correlation with the time points considered [64]. In fact, it found that NPs were preferentially located in the connective tissue and in the capillary lumen at 1 and 24 h after exposure, respectively. The epithelium and the connective tissues were not deposition sites but only passageways for NPs to reach the capillary circulation. However, whether NPs can translocate from air-filled spaces to the systemic circulation is still not fully understood, and further investigation is needed.

2.1.3. Carcinogenic Effects. Animal and human epidemiological data have led TiO_2 to be designated as "possibly carcinogenic" to humans [28, 29, 89]. Surprisingly, however, few studies have investigated the carcinogenicity of TiO_2 NPs, and only recently, NIOSH concluded that inhaled ultrafine TiO_2 is a potential occupational carcinogen [15]. The most relevant data for assessing the health risk for workers are results from a chronic animal inhalation study performed by Heinrich et al. [56], in which rats exposed by inhalation to P25 Degussa TiO_2 NPs showed increased rates of adenocarcinomas compared to controls. Interestingly, in this study, mice exposed to the same NPs, according to the same methodology, did not show differences in tumor rates compared to controls. Aside from demonstrating that TiO_2 NPs can induce lung cancer in exposed animals, an interesting finding of this work is the difference in carcinogenicity between rats and mice. The species difference in response to insoluble and low toxicity dust, and the controversial approach to classify such a dust as a potential human carcinogen is subject to debate. Moreover, NIOSH has concluded that TiO_2 is not a direct-acting carcinogen, but acts through a secondary genotoxic mechanism primarily related to particle size and surface area [15], as supported by the comprehensive analysis of the data reported by Heinrich et al. [56] and those obtained by Lee et al. with fine-sized TiO_2 [90]. However, considering the pressing concern regarding cancerogenic effects of TiO_2 NP exposure on general and occupational populations and the limited number of *in vivo* studies on this topic, additional researches seem highly necessary.

2.2. Nervous System. Regarding TiO_2 NP neurotoxicity, different studies have demonstrated the ability of these NPs to translocate into the brain, irrespective of the route of exposure [31, 73, 91–94], the form of the TiO_2, and the size of the NPs and to accumulate in this organ [31, 91–93] inducing numerical and structural changes in the neuronal architecture [31, 92, 93] (Table 2).

Several studies have unequivocally showed that rutile TiO_2 NPs, taken up by intranasal exposure, could be translocated into the central nervous system (CNS) via the olfactory pathway in mice and accumulate in the entire brain, mainly in the hippocampus regions [31, 91, 92]. However, to verify the generalization of this phenomenon, further studies are needed in other animal species. Regardless, this neuronal translocation pathway should be taken into account for health risk assessments of TiO_2 NPs. In particular, it could be useful to avoid possible toxic effects to the general population but principally to subjects that work with these NPs. Brain accumulation was also demonstrated in mice intratracheally instilled with no characterized TiO_2 NPs able to penetrate alveolar-capillary barrier, enter blood circulation, and further penetrate blood-brain barrier inducing tissue damage [73].

The deposition of TiO_2 NPs in brain was reported to induce changes in the release and metabolism of neurotransmitters, although with the same conflicting results, maybe due to the different route of exposure [31, 91, 93, 94]. Particularly, the levels of norepinephrine (NE) and 5-hydroxytryptamine (5-HT) increased after intranasal exposure [91] while decreased in response to intragastric administrations of anatase TiO_2 NPs [93]. Dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic (HVA), and 5-hydroxyindole acetic acid (5-HIAA) contents were reduced by both types of treatment [91, 94]. Moreover, intranasal instillation of rutile [31] and intragastric administrations of anatase TiO_2 NPs [94] increased catalase and acetylcholinesterase (AchE) activity, soluble protein carbonyl, acetylcholine (Ach), glutamic acid (Glu), and NO content. Intraperitoneally injected anatase TiO_2 NPs increased NO, while decreased Glu content and AchE activity [92]. Interestingly, the study performed by Hu et al. [94] demonstrated also that the contents of Ca, Mg, Na, K, Fe, and Zn in brain were significantly altered after TiO_2 NP exposure. The disturbed homeostasis of trace elements, neurotransmitters, and enzymes in brain may be responsible of the spatial recognition memory impairments reported in treated mice [94]. However, as assessed by a proteomic analysis, proteins of the mouse brain resulted differentially expressed in response to TiO_2 NP treatment even if NPs were not detected in the tissue [95].

As observed *in vitro* [96–98], oxidative stress-related damage with a consequent alteration in the balance between oxidative and antioxidative activities was also reported in *in vivo* studies [31, 73, 92, 93, 95]. Malondiadehyde (MDA) levels increased after intranasal instillations [31, 92], intraabdominal injections [93], and intratracheal instillation in mice [73]. Similarly, Li et al. [73], and Ma et al. [93] demonstrated that the level of superoxide anion (O_2^-), peroxide (H_2O_2) [73, 93] and hydroxyl radicals [73] increased in treated animals. TiO_2 NPs are able also to induce an inflammatory effect in brain of treated mice, as detected by the increased cytokine release [92, 99]. In the latter study, rutile and P25 Degussa TiO_2 NPs intraperitoneally injected in mice after lipopolysaccharide (LPS) increased the mRNA levels of IL-1β and tumor necrosis factor (TNF)-α and also those of IL-1β protein. Interestingly, LPS induction was necessary to cause this phenomenon, suggesting the importance of a trigger element or of a possible synergistic effect in tissue responses to TiO_2 NPs.

Finally, several studies demonstrated the embryotoxic role of maternal intravenous injection of no characterized TiO_2 NPs [100], or subcutaneous injections of anatase TiO_2 NPs [101–103]. Following this latter treatment, TiO_2 accumulation was detected in the offspring cerebral cortex and olfactory bulb and numerous olfactory bulb cells resulted positive to markers of apoptosis [101]. Moreover, the same prenatal exposure induced an altered expression of genes involved in brain development, cell death, and the response to oxidative stress in the newborn pups [102]. Finally, the influence of prenatal TiO_2 NP exposure on the dopaminergic system was demonstrated by the increased DA, DOPAC, HVA and 3-methoxytyramina hydrochloride levels in the prefrontal cortex and in the neostriatum of exposed mice [103].

These data confirm that TiO_2 NPs can be transferred from mothers to the fetal brain, inducing toxic effects on fetal brain development carrying a series of nervous system disorders. In this context, a deep knowledge regarding the influence of TiO_2 NPs on neuronal cells is becoming urgent. Though additional studies are necessary to support these findings, they should be taken in consideration for the correct risk assessment of TiO_2 NP exposure, particularly during a complex biological status like pregnancy and the early stage of life.

2.3. Dermal and Mucosal System. Studies regarding the effects of TiO_2 NPs on the dermal system (in particular, the percutaneous absorption of NPs) show more homogeneous findings, demonstrating a clear absence of penetration through the intact epidermal barrier when TiO_2 NP formulations are applied on different animal [104, 105] and human [106–110] skins (Table 3). Considering the widespread use of TiO_2 NPs in cosmetic sunscreens (due to their broad-spectrum UV absorption and high esthetical acceptance) [107], this is an important finding.

Sadrieh et al. [104] analyzed the correlation between different forms of TiO_2 and percutaneous absorption in micropig skin, demonstrating that irrespective of the form, after a subchronic exposure, NPs were found in the stratum corneum but not in the deeper epidermal strata. Anatase TiO_2 NPs subacutely exposed to hairless rat skin were only detected in the horny layer of the interfollicular epidermis, without penetration into viable cell layers or induction of any cellular changes [111].

Neither the shape [104, 105, 107, 108] nor the surface chemistry [107–109] seems to influence NP penetration after acute and subacute exposure.

Table 2: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on nervous system.

References	Crystal phase composition (particle size in nm)	Nervous system Type of exposure	Type and number of animals	Results
Wang et al., 2007 [91]	TiO_2 (25, 80, 155)	**Intranasal instillation**: 50 mg/kg TiO_2 BW every other day for 20 days.	Female CD mice	**Ti brain content:** increased. **Neurotransmitters**: NE and 5-HT increased; DA, DOPAC, HVA, and 5-HIAA decreased.
Wang et al., 2008 [31]	Rutile TiO_2 (80) Anatase TiO_2 (155)	**Intranasal instillation:** 500 μg TiO_2 every other day for 15 times.	15 female CD-1(ICR) mice	**Ti brain distribution:** mainly in olfactory bulb and hippocampus. **Oxidative stress:** CAT, MDA, Pr. Carb. increased, SOD decreased. **Neurotransmitters:** AchE, Glu and NO increased.
Wang et al., 2008 [92]	See Wang et al., 2008 [31]	**Intranasal instillation**: 500 μg TiO_2 every other day for 15 times.	10 female CD-1(ICR) mice	**Ti brain distribution:** mainly in hippocampus. **Oxidative stress:** GSH-Px, GST, SOD, GSH, and MDA increased. **Histology:** irregular neuronal arrangement, condensated chromatin. **Inflammatory action:** increased TNF-α and IL-1β levels.
Shimizu et al., 2009 [102]	Anatase TiO_2 (25–70)	**Subcutaneous injections**: 100 μL of TiO_2 at 1 μg/μL on gestational days 6, 9, 12, and 15.	15 pregnant ICR mice, 21 male fetuses and pups	**Gene expression**: up-regulated cell death, apoptosis, brain development, oxidative stress, apoptosis, neurotransmitter genes.
Takeda et al., 2009 [101]	Anatase TiO_2 (25–70)	**Subcutaneous injections**: 100 μL of TiO_2 at 1 mg/mL at 3, 7, 10 and 14 days post-coitum.	6 pregnant Slc:ICR mice per group	**TiO_2 offspring brain distribution:** cortex and olfactory bulb. **Apoptosis:** presence of markers and features in olfactory cells.
Takahashi et al., 2010 [103]	Anatase TiO_2 (25–70)	**Subcutaneous injections**: 100 μL of TiO_2 at 1 mg/mL at gestational days 6, 9, 12, 15, 18.	Pregnant Slc:ICR mice per group	**Monoamine levels:** DA, DOPAc, HVA, 3-MT increased in the prefrontal cortex and neostriatum.
Ma et al., 2010 [93]	Anatase TiO_2 (5) Bulk TiO_2 (10–15 nm.)	**Intra-abdominal injections:** 5–150 mg/kg nano-TiO_2 and 150 mg/kg bulk TiO_2 every day for 14 days, respectively.	10 female CD-1(ICR) mice per group	**Ti brain content**: higher increase with nano TiO_2. **Oxidative stress**: O_2^-, H_2O_2, MDA, NOS, iNOS, NO increased; antioxidative enzymes, GLU, AchE decreased. **Histology**: filamentous-shaped neurons and inflammatory cells.
Shin et al., 2010 [99]	Rutile TiO_2 (1 μm) P25 TiO_2 (21)	**Intraperitoneal injections:** 40 mg/kg TiO_2 30 min after vehicle or 5 mg/kg LPS.	3–6 male C57BL/6 mice per group	**Inflammatory action:** after LPS, TiO_2 NPs increased IL-1β, TNF-α and iNOS and induced microglial activation. **Oxidative stress:** after LPS TiO_2 NPs enhanced ROS.

Table 2: Continued.

References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
		Nervous system		
Hu et al., 2010 [94]	Anatase TiO_2 (5)	**Intragastric administration**: 5–50 mg/kg TiO_2 suspension every day for 60 days.	20 female mice per group	**Neurotransmitters:** ACh, Glu, and NO increased; NE, DA, DOPAC,5-HT, and 5-HIAA decreased. **Enzyme activity:** decreased Na^+/K^+, Ca^{2+}, Ca^{2+}/Mg^{2+} ATPase; promoted AchE, and iNOS.
Li et al., 2010 [73]	Anatase TiO_2 (3)	**Intratracheal instillation:** 3.3 mg/kg TiO_2 once a wk for 4 wks.	13 male Kunming mice per group	**Ti brain content**: increased **Oxidative stress:** O_2^-, OH^-, H_2O_2, MDA increased in brain. **Histology:** exudates, inflammatory infiltration and necrosis.
Yamashita et al., 2011 [100]	TiO_2 (35)	**Intravenous injection:** 0.8 mg TiO_2 for 2 consecutive gestational days.	Pregnant mice	**Ti distribution:** TiO_2 detected in fetal brain
Jeon et al., 2011 [95]	TiO_2	N.A	N.A.	**Proteomic analysis:** altered protein expression. **Oxidative stress:** antioxidant and AchE activities reduced.

5-HIAA, 5-hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine; 3-MT, 3.methoxytyramine-hydroclhoride; Ach, acetylcholine; AchE, acetylcholinesterase; CAT catalase activity; NE, norepinephrine; DA, dopamine; DOPAC, 3, 4-dihydroxyphenylacetic acid; Glu, glutamic acid; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GST, glutathione-S-transferase; HVA, homovanillic acid; IL-, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MDA, malondialdehyde; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; Pr.Carb., Protein carbonyls; ROS, reactive oxygen species; SOD, superoxide dismutase; TiO_2 NPs, titanium dioxide nanoparticles; TNF-α, tumor necrosis factor-α.

Table 3: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on dermal system.

References	Crystal phase composition (particle size in nm)	Dermal and mucosal system Type of exposure	Type and number of animals/subjects	Results
Tan et al., 1996 [112]	Sunscreen containing 8% microfine TiO_2	**Cutaneous application:** 2 times/day for 2–6 wks.	13 male and female volunteer patients	**TiO_2 absorption:** levels of Ti in dermis were higher in treated patients compared to controls, but not significantly.
Lademann et al., 1999 [113]	W/O emulsion UV-Titan M 160	**Cutaneous application:** 2 mg/cm^2 on the volar forearm, 5 times on the first 3 days, and once on the 4th.	Human volunteers	**TiO_2 absorption:** mostly located in the upper st. corneum.
Pflücker et al., 2001 [107]	W/O emulsion T805 coated with trimethyl octylsilane TiO_2 (20) W/O emulsion Eusolex T-2000 TiO_2 coated with Al_2O_3 and SiO_2 (10–15) W/O emulsion Tioveil AQ TiO_2 coated with Al and SiO_2 (100)	**Cutaneous application:** 4 mg/cm^2 on forearm skin for 6 hr.	Human volunteer	**TiO_2 absorption:** TiO_2 solely localized on the outermost surface of the st. corneum, not in the deeper subcutaneous layers.
Schulz et al., 2002 [108]		See Pflücker et al., 2001 [107]		**TiO_2 absorption:** located on the outermost surface of the st. corneum and not in the deeper subcutaneous layers.
Menzel et al., 2004 [105]	Sunscreen containing TiO_2 (length: 45–150; width: 17–35)	**Cutaneous application:** on the back pig skin for 8–48 hr.	Pigs	**TiO_2 absorption:** TiO_2 penetrated through intercellular spaces of the st. corneum into the st. granulosum.
Kertész et al., 2005 [106]	Hydrophobic emulsion TiO_2 NPs.	**Cutaneous application:** on human foreskin grafts for 1–48 hr.	Human grafts transplanted into SCID mice	**TiO_2 absorption:** corneocyte layers of the st. corneum.
Mavon et al., 2007 [109]	Emulsion with 3% T805 Degussa TiO_2 coated with trimethyl octylsilane (20)	**Cutaneous application:** 2 mg/cm^2 on upper arms.	3 human volunteers	**TiO_2 absorption:** 93% of the TiO_2 applied was recovered in the st. corneum.
Kiss et al., 2008 [110]	Hydrophobic emulsion containing micronized TiO_2.	**Cutaneous application:** 2 mg/cm^2 on human foreskin grafts for 24 hr.	Human grafts transplanted into SCID mice	**TiO_2 absorption:** outermost layers of the st. corneum.
Yanagisawa et al., 2009 [114]	Rutile TiO_2 (15, 50, 100)	**Intradermal injections:** of 20 μg TiO_2 with or without mite allergen on the ears.	11 male C/NgaTndCrj (NC/Nga) mice per group	**Atopic dermatitis:** allergen + TiO_2 enhanced ear thickening. **Inflammatory action:** allergen + TiO_2 increased EOSs, IL-4, MCs and decreased IFN-γ; TiO_2 increased IL-13.
Wu et al., 2009 [115]	Anatase TiO_2 (5 ± 1, 10 ± 1) Rutile TiO_2 (25 ± 5, 60 ± 10) P25 Degussa TiO_2 (~21) TiO_2 (0.3–0.5 μm)	**Cutaneous application**: 24 mg TiO_2 formulations on the pig ear for 30 days and on the mouse interscapular skin for 60 consecutive days.	3 male reared pigs per group 6 hairless mice (BALB/c/nu/nu) per group	**TiO_2 absorption in pigs:** TiO_2 detected in the st. corneum, granulosum, prickle and basal cell layer, not in the dermis; **TiO_2 absorption in mice:** increased MDA, reduced HYP content, and excessive keratinisation in skin.

Table 3: Continued.

References	Crystal phase composition (particle size in nm)	Dermal and mucosal system Type of exposure	Type and number of animals/subjects	Results
Sadrieh et al., 2010 [104]	P25 Degussa TiO_2 (30–50) Rutile TiO_2 coated with aluminium hydroxide/dimethicone copolymer (diameter: 20–30, length: 50–150) Submicron TiO_2 (300–500)	**Cutaneous application:** 2 mg cream/cm^2 skin for 4 times/day, 5 days/week, 4 weeks of.	3 female Yucatan minipigs per group	**TiO_2 absorption:** TiO_2 detected in the upper st. corneum and follicular lumen, with few particles observed in dermal layers as a contamination results.
Adachi et al., 2010 [111]	W/O emulsion containing anatase TiO_2 (26.4 ± 9.5)	**Cutaneous application:** 4 mg/cm^2 TiO_2 on dorsal skin.	Male hairless Wistar Yagi rats	**TiO_2 absorption:** TiO_2 detected in the horny layer of the interfollicular epidermis.
Moon et al., 2011 [116]	TiO_2 (<25; <100)	**Intraperitoneal injections:** once a day for 28 consecutive days before subcutaneous implantation with B16F10 melanoma cells.	Mice	**Tumor growth:** increased
Furukawa et al., 2011 [117]	Coated TiO_2 (long axis: 50–100; short axis: 10–20)	**Cutaneous application**: 5–20 mg TiO_2 in the postinitiation phase in a skin carcinogenesis model.	10–20 CD1 (ICR) female mice per group	**Carcinogenicity:** no increased development of skin nodules

EOS, eosinophil; HYP, hydroxyproline; IL-, interleukin; IFN-γ, interferon- γ; MC, mast cell; MDA, malondialdehyde; TiO_2 NPs, titanium dioxide nanoparticles.

Pflücker et al. [107] demonstrated that all TiO_2 NP types applied on skin areas of human forearm were only detected on the outermost layer of the stratum corneum, irrespective of their surface chemistry, NP size, and shape. The same results were confirmed by Schulz et al. [108]. Similarly, after application of lanceolate TiO_2 NPs on the backs of pigs, high concentrations of Ti were found in the stratum corneum and granulosum, but not into the stratum spinosum [105].

Mavon et al. [109] demonstrated that 93% of the TiO_2 T805 NPs hydrophobically coated with trimethyloctylsilane applied on human upper arms were found in the stratum corneum, decreasing continuously with depth. No TiO_2 penetration into viable skin layers was assessed confirming the *in vitro* findings of the study.

The studies investigating the Ti penetration and distribution in biopsies obtained from human skin grafts transplanted into severe combined immune deficiency (SCID) mice could only detect Ti in the outermost layers of the stratum corneum of the skin biopsies, irrespective of the various NP formulation applied and the time points considered (1–48 h) [106, 110].

As also confirmed by Menzel et al. [105], Lademann et al. [113] failed to demonstrate a role for hair follicles as a percutaneous absorption pathway. In fact, Ti was detected in the stratum corneum and in the follicle channels but not in the interfollicular space under the stratum corneum or into the viable layers of the forearm skin of human volunteers.

Only a few reports detail Ti penetration into human dermis [112, 115]. For instance, in a pilot study conducted in 1996, Tan et al. [112] demonstrated percutaneous penetration of Ti in patients subchronically applied sunscreen containing microfine TiO_2. Although Ti levels in the dermis of exposed subjects were higher than controls, these results were not statistically significant and were not confirmed by subsequent studies. Moreover, Wu et al. [115] reported the ability of TiO_2 NPs in different form, anatase, rutile, P25 Degussa, and different sizes to penetrate through the skin, reaching different tissues (e.g., liver, spleen, and heart) where pathological lesions were induced after subchronic dermal exposure of hairless mice. Moreover, increased MDA levels were determined in the skin, which also showed excessive keratinization, thinner dermis and wrinkled epidermidis. These data could not be confirmed when reared pigs were treated with two types of the previously employed NPs for shorter periods of time.

The same interesting points of discussion emerged from this latter study. It seems that the penetrative ability of similar TiO_2 NPs into the cutaneous layers depends on the animal species studied and on the period of exposure. Furthermore, pathological skin lesions due to NP application are likely ascribed to oxidative stress, which was also detected on human and murine dermal fibroblasts *in vitro* [118–120].

Intradermal injection of TiO_2 NPs aggravated atopic dermatitis (AD) symptoms related to mite allergen in mice assumed to show skin barrier dysfunction/defect through Th-2 immune inflammatory responses and histamine release [114]. These data support the importance of an intact dermal defense system to prevent uptake of TiO_2 NPs, which can lead to skin damage, activation of the immune system, and eventually a decrease in the allergy threshold [105].

Ultimately, the importance of the human studies on percutaneous TiO_2 absorption is underlined by the fact that these experiments alone are able to provide a relevant approach to direct human risk assessment and are the first step in the complex process of generalizing implications regarding environmental or occupational health.

Regarding skin carcinogenicity, conflicting results were reported [116, 117]. Tumor growth was increased when mice were intraperitoneally injected with TiO_2 NPs prior to the subcutaneous implantation of B16F10 melanoma cells [116]. The authors suggest that TiO_2 NPs might have the potential to enhance tumor growth *in situ* through immunomodulation of B- and T-lymphocytes, macrophages, and NK cells as supported also by results obtained in the spleen tissue. In contrast to these findings, Furukawa et al. [117] investigated the safety of coated and uncoated TiO_2 NPs in a mouse medium-term skin carcinogenesis bioassay. Both TiO_2 NPs applied to mouse skin in the postinhitiation phase did not increase the development of skin nodules histopathologically diagnosed as squamous cell hyperplasia, sebaceous gland hyperplasia, squamous cell papilloma, and keratoacanthoma. According to these data, TiO_2 NPs do not possess postinhitiation potential and there is no carcinogenic risk relevant to percutaneous application of TiO_2 NP preparations.

2.4. Cardiovascular System. Due to the complexity of the cardiovascular system, studies regarding the potential effects of TiO_2 NPs on this system have focused on different functional aspects, such as cardiotoxicity [121, 122], cardiovascular parameters, such as systolic blood pressure (SBP) and heart rate (HR) variability [79], induction of thrombosis [22, 123], and the alteration of vasomotor responses [124–128] (Table 4).

TiO_2 NPs are able to induce high LDH, creatine kinase (CK), alpha-hydroxybutyrate dehydrogenase (HBDH), and aspartate aminotransferase (AST) activities used as markers of myocardial lesions, irrespective of the form of TiO_2, anatase [122] or a mixture of anatase and rutile [129], or the route of exposure, intraperitoneal injections in mice [122], or oral gavage in rats [129]. Moreover, acute cardiotoxicity, in terms of higher serum LDH and alpha-HBDH enzymes, was demonstrated after a single oral gavage of TiO_2 NPs administered to mice [121]. Unfortunately, the lack of data regarding the LDH isoform prevents a clear correlation between these increases and cardiac insults. Moreover, the study performed by Wang et al. [121] did not provide information on TiO_2 form, and consequently, it does not allow comparisons with the two previous described works [122, 129].

When intratracheally instilled in rats, rutile Fe-doped TiO_2 nanorods significantly increased SBP and HR in treated animals, which could be the consequence of the systemic inflammation induced by the same particles [79].

Regarding the TiO_2 NP prothrombotic effect, the currently available data are conflicting. For instance, Chen et al. [22] observed thrombosis in the pulmonary vascular system

Table 4: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on cardiovascular system.

		Cardiovascular system		
References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
Wang et al., 2007 [121]	TiO_2 (25, 80, 155)	**Intragastric administration**: 5 g/kg TiO_2 in a minute.	20 male and female CD-1 (ICR) mice per group	**Biochemical parameters:** 80 and 25 nm TiO_2 increased LDH and alpha-HBDH compared to controls and fine group.
Chen et al., 2009 [22]	Anatase TiO_2 (80–110)	**Intraperitoneal injections**: 32–2592 mg/kg TiO_2.	10 male and female ICR mice per group	**Vascular system:** pulmonary thrombosis
Liu et al., 2009 [122]	Anatase TiO_2 (5) Bulk rutile TiO_2 (10–15 μm)	**Intra-abdominal injections**: 5–150 mg/kg BW anatase TiO_2 and 150 mg/kg bulk TiO_2 everyday for 14 days.	10 female CD-1 (ICR) mice per group	**Biochemical parameters:** CK, LDH, AST, and alpha-HBDH were increased by both TiO_2.
Bu et al., 2010 [129]	Rutile-anatase TiO_2 mixture (<50)	**Intragastric administration:** 0.16–1 g/kg TiO_2 once a day for 14 consecutive days.	16 male and female Wistar rats per group	**Biochemical parameters:** increased CK and LDH.
Bihari et al., 2010 [123]	Rutile TiO_2 ($\sim 10 \times 40$)	**Intravenous administration:** 1 mg/kg TiO_2 10 min before thrombosis induction.	8 male C57BL/6Ncrl mice	**Mesenteric and cremasteric thrombosis:** not determined.
Nurkiewicz et al., 2008 [124]	P25 anatase-rutile TiO_2 (21) TiO_2 (1 μm)	**Inhalation:** 1.5–20 mg/m^3 TiO_2 for 120–720 min.	7–12 male Sprague Dawley rats per group	**Spinotrapezious arteriolar endothelium dilation:** impaired by both TiO_2.
Nurkiewicz et al., 2009 [125]	P25 anatase-rutile TiO_2 (21) Rutile TiO_2 (1 μm)	**Inhalation:** 1.5–16 mg/m^3 TiO_2 for 240–720 min.	8–16 male Sprague Dawley rats per group	**Spinotrapezious arteriolar endothelium dilation:** impaired by both TiO_2.
LeBlanc et al., 2009 [126]	P25 anatase-rutile TiO_2 (21)	**Inhalation**: 6 mg/m^3 TiO_2 for 240 min.	26 male Sprague Dawley rats	**Coronary arteriolar endothelium:** TiO_2 increased spontaneous arteriolar tone and impaired flow and vasodilator induced dilation.
LeBlanc et al., 2010 [127]	P25 anatase-rutile TiO_2 (21)	**Inhalation**: 6 mg/m^3 TiO_2 for 240 min.	50 male Sprague Dawley rats	**Coronary arteriolar endothelium dilation:** impaired by TiO_2. **Oxidative stress:** ROS increased in coronary microvascular walls.
Courtois et al., 2010 [128]	P25 Degussa TiO_2 (15)	**Intratracheal instillation**: 100 μg TiO_2 in 0.5 mL saline.	Male Wistar or Sprague Dawley rats	**Intralobar arteries vasomotor responses to PGF2α, KCl, Ach:** not altered.
Nemmar et al., 2011 [79]	Rutile Fe-doped TiO_2 (length: 80; diameter: 7)	**Intratracheal instillation:** 1, 5 mg/kg TiO_2	4 male Wistar rats per group	**Cardiovascular parameter:** HR and SBP increased

Ach, acetylcholine; Alpha-HBDH, alpha-hydroxybutyrate dehydrogenase; AST, aspartate aminotransferase; CK, creatine kinase; HR, heart rate; KCl, Potassium chloride LDH, lactate dehydrogenase; PGF2α, prostaglandin F2α; ROS, reactive oxygen species; SBP, systolic blood pressure; TiO_2 NPs, titanium dioxide nanoparticles.

of mice treated via intraperitoneal injection with anatase TiO_2 NPs. On the contrary, Bihari et al. [123] failed to confirm thrombosis induction in both the mesenteric and cremasteric circulation of mice given parentally rutile TiO_2 NPs. The higher exposure dose used by Chen et al. [22] compared to that of Bihari et al. [123], up to 2592 mg/kg *versus* 1 mg/kg, respectively, may be responsible for the induction of thrombosis ascribed to the blockage of blood vessels by TiO_2 particles. Moreover, the difference in the form of TiO_2 used by these two studies may explain some differences in the results. However, the very limited number of *in vivo* and *in vitro* studies on this regard does not support such conclusions. Thus, further studies are necessary to confirm these results and to investigate the mechanism(s) leading to thrombus formation.

The vasomotor response of the spinotrapezious muscle arterioles is impaired by P25 Degussa TiO_2 NP inhalation [124, 125]. This vascular impairment was due to a dose-dependent reduction in NO endothelium production induced by microvascular oxidative and nitrosative stress [125]. Local ROS production may consume endothelium-derived NO or compromise NO endogenous production leading to vascular dysfunction as confirmed by a partially restored NO production by radical scavenging.

The loss of microvascular vasodilator capacity can significantly influence the normal homeostasis in any organ, causing impairment of tissue perfusion and compromising organ function. In this context, a further study by the same group demonstrated that after TiO_2 NP exposure, coronary arteriole response to vasodilators was impaired though with conserved responsiveness to NO [126]. As previously detailed, the role motive of the increased microvascular ROS production was confirmed by the restored impairment after incubation with ROS scavengers [127].

Finally, vasomotor responses of intralobar pulmonary arteries removed from rats intratracheally exposed to Degussa TiO_2 NPs were not altered in a study carried out by Courtois et al. [128]. The *ex vivo* contractile response to prostaglandin F2α and KCl and the relaxant response to Ach were not altered by TiO_2 NP treatment [128, 130].

However, without appropriate particle characterization, it is difficult to compare findings between toxicological studies. Future studies are necessary to provide deeper knowledge regarding the toxic effects of TiO_2 NPs on the cardiovascular system.

2.5. Liver. The toxic effects of TiO_2 NPs on liver function were demonstrated in several studies through the detection of increases of AST, alanine aminotransferase (ALT) [21, 22, 79, 121, 122, 129, 131–133], ALP, pseudocholinesterase (PChE), leucine acid peptide (LAP), TP, ALB [21, 40, 122, 132, 133], and globulin (GLB) levels. Decreased ALB/GLB ratios [21, 132] and increased ALT/AST ratios, a more sensitive indicator of hepatic injury [121, 131, 134], have also been detected (Table 5).

These results were obtained irrespective of the form of TiO_2, particularly anatase [22, 40, 115, 122, 132–135], rutile [79, 115] and P25 Degussa TiO_2 NPs [115]; the NP different size [115, 121] and shape [79]; the modified surface chemistry of the particles [79].

Other studies demonstrated TiO_2 NP hepatotoxicity effects, in terms of increased ALT levels and ALT/AST ratio both after a single oral administration [121] or repeated intraperitoneal treatments in mice [122], unfortunately, without reporting details regarding the form of TiO_2 used.

Moreover, the route of exposure, such as the intragastric [21, 121, 129, 133, 136], intraperitoneal [22, 122, 131, 132], intratracheal [40, 75]; dermal [115], or intraarticular [134], does not seem to determine different toxic effects on liver function, both in terms of type and entity of manifestation. The intraarticular administration was used by Wang et al. [134] to simulate the release of NPs into joint cavities from the nanocoated surface of prostheses considering the good prospects for application of nanomaterials in prosthetic implants.

Interestingly, anatase, rutile, and P25 Degussa TiO_2 NPs were able to penetrate the dorsal skin of hairless mice and to accumulate in the liver inducing oxidative stress, and focal necrosis in the parenchyma [115]. The potential role of an oxidative attack in causing liver damage was recently demonstrated in mice intragastrically [136] and intraperitoneally [135] exposed to nanoparticulate anatase TiO_2. ROS accumulation, lipid peroxidation, and altered expression of genes involved in antioxidative or detoxification processes were triggered by NP treatment [136].

Interesting results were obtained by Li et al. [137] analysing the interactions between anatase TiO_2 NPs and the DNA extracted by the liver of mice intraperitoneally exposed. They reported that nanoanatase TiO_2 could be inserted into DNA base pairs, bind to DNA nucleotide, and alter the secondary structure of DNA. Moreover, NPs could cause liver DNA cleavage and hepatocyte apoptosis [137].

Several studies also detected signs of hepatic damage in terms of histopathological changes and hepatocyte ultrastructural alterations that could lead to impaired liver function [21, 22, 115, 121, 132, 134], while only few did not confirm these results [30, 129]. Particularly, hepatic fibrosis hydropic [22, 121] and fatty [133, 134] degeneration of hepatocytes, prominent vasodilatation [21, 132], and focal ischemia were induced by TiO_2 NP treatment [132]. Necrotic [22, 121, 133] and apoptotic cells [132, 133, 136] were detected, and infiltration of inflammatory cells was also found [22, 79, 132–134]. The inflammatory reaction in response to NP insult was also confirmed by significant increase of both mRNA and protein expression levels of several inflammatory cytokines and mediators in liver of treated mice [132, 133].

Regarding the metabolic homeostasis of mice treated with TiO_2 NPs, several studies demonstrated increased contents of triglycerides (TG), total cholesterol (TCHO) [21, 122, 132], high-density lipoprotein cholesterol (HDL-C) [122, 132], and glucose [122] compared to controls, while low-density lipoprotein cholesterol (LDL-C) resulted lower [132].

Moreover, recent metabonomic studies, demonstrated the ability of TiO_2 NPs to cause a series of changes in endogenous metabolite levels in the ^{1}H nuclear magnetic

Table 5: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on liver.

References	Crystal phase composition (particle size in nm)	Liver Type of exposure	Type and number of animals	Results
Wang et al., 2007 [121]	TiO_2 (25, 80, 155)	**Intragastric administration**: 5 g/kg TiO_2 in a minute.	20 male and female CD-1 (ICR) mice per group	**Biochemical parameters:** ALT and ALT/AST increased. **Histology:** hydropic degeneration and necrosis.
Fabian et al., 2008 [138]	Anatase-rutile TiO_2 mixture (20–30)	**Intravenous injection:** 5 mg/kg	3 male Wistar rats per group	**Biochemical parameters:** no alterations in TBIL, ALP, ALT, AST.
Liang et al., 2009 [30]	TiO_2 (5, 21)	**Intratracheal instillation**: 0.5–50 mg/kg TiO_2.	6 male and female Sprague Dawley rats per group	**Biochemical parameters:** no changes in TP, ALB, ALT, AST. **Oxidative stress:** decreased SOD and increased MDA activity.
Guo et al., 2009 [131]	N.A.	**Intraperitoneal injections**: 200 and 500 mg/kg TiO_2 every other day for 5 times.	15 male ICR mice per group	**Biochemical parameters:** ALT and AST/ALT increased.
Chen et al., 2009 [22]	Anatase TiO_2 (80–110)	**Intraperitoneal injections**: 324–2592 mg/kg TiO_2.	10 male and female ICR mice.	**Biochemical parameters:** ALT and AST increased. **Histology:** fibrosis, hydropic degeneration, necrotic, and apoptotic cells, NEUs were detected.
Liu et al., 2009; 2010 [122, 135]	Anatase TiO_2 (5) Bulk rutile TiO_2 (10–15 μm)	**Intra-abdominal injections**: 5–150 mg/kg BW anatase TiO_2 and 150 mg/kg bulk TiO_2 everyday for 14 days.	10 female CD-1 (ICR) mice per group	**Biochemical parameters:** ALT, ALP, ALB, glucose, TG, TCHO, HDL-C levels were increased by both particles; LAP, PChE, TP, were increased and TBIL was reduced by anatase TiO_2. **Oxidative stress:** induced by anatase TiO_2 in liver
Ma et al., 2009 [132]		See Liu et al., 2009 [122]		**Biochemical serum parameters:** ALT, ALP, AST, LDH, PChE, LAP, TCHO, and HDL-C increased by both particles; ALB, GLB, TG increased and LDL-C decreased by anatase TiO_2. **Histology:** basophilia, ischemia and vein congestion (both TiO_2). Apoptosis induced by anatase TiO_2. **Inflammatory action:** NF-kB, MIF, TNF-α, IL-6, IL-1β, CRP, IL-4, and IL-10 increased by anatase TiO_2.
Wu et al., 2009 [115]	Anatase TiO_2 (10 ± 1) Rutile TiO_2 (25 ± 5; 60 ± 10) P25 Degussa TiO_2 (~21) TiO_2 (0.3–0.5 μm)	**Cutaneous application**: 24 mg of 5% TiO_2 test formulation on the dorsal interscapular skin	6 hairless mice (BALB/c/nu/nu) per group	**Liver histology:** TiO_2 penetrated the skin inducing necrosis. **Oxidative stress:** increased MDA activity in liver.

Table 5: Continued.

References	Crystal phase composition (particle size in nm)	Liver Type of exposure	Type and number of animals	Results
Wang et al., 2009 [134]	Anatase TiO_2 (diameter: 45.87 ± 7.75; thickness:10–15)	**Intraarticular injection**: 0.2–20 mg/kg TiO_2 in the knee joints every other day for 4 times.	10 male Sprague Dawley rats per group	**Biochemical parameters:** ALP decreased; AST/ALT, LDH increased. **Histology:** fatty degeneration, inflammatory cell infiltration.
Duan et al., 2010 [21]	Anatase TiO_2 (5)	**Intragastric administration**: 62.5–250 mg/kg TiO_2 for 30 consecutive days.	20 female CD-1 (ICR) mice per group	**Biochemical parameters:** ALT, ALP, AST, LDH, ChE, TP, TG, TCHO increased, ALB/GLB, TBIL decreased. **Histology:** blurred hepatocytes, congested vessels.
Cui et al., 2011 [133]	Anatase TiO_2 (5)	**Intragastric administration**: 5, 10, 50 mg/kg TiO_2 for 60 consecutive days.	20 female CD-1 (ICR) mice per group	**Biochemical parameters:** ALT, AST, ALP, LDH, PChE, LAP increased. **Inflammatory action:** IkB and IL-2 decreased; IKK1, IKK2, NF-kB, NF-kBP52–65, TNF-α, NIK, TLR-2, and TLR-4 increased. **Histology:** fatty degeneration, necrosis, apoptosis, inflammation.
Cui et al., 2010 [136]	Anatase TiO_2 (6.9)	**Intragastric administration**: 5, 10, 50 mg/kg TiO_2 for 60 consecutive days.	20 female CD-1 (ICR) mice per group	**Histology:** hepatocyte apoptosis. **Oxidative stress**: O_2^-, H_2O_2, MDA, NO increased. **Gene expression:** SOD, CAT, GSH-Px, MT, GST, HSP70, P53, TF decreased, CYP1A increased.
Bu et al., 2010 [129]	Rutile-anatase TiO_2 mixture (<50)	**Intragastric administration**: 0, 0.16, 0.4, 1 g/kg TiO_2 once a day for 14 consecutive days.	16 male and female Wistar rats per group	^{1}**H NMR urine analysis**: increase in α-ketoglutarate, hippurate, histidine, TMAO, taurine, citrulline, acetate, PAG, and citrate levels; decrease in methionine and 3-D-HB levels ^{1}**H NMR serum analysis**: increase in TMAO, choline creatine, 3-D-HB, phosphocholine; decrease in glutamate, acetoacetate, glutathione, methionine, glutamine, and pyruvate. **Histology:** no alterations.
Li et al., 2010 [137]	Anatase TiO_2	**Intra-abdominal injections:** 5, 10, 50, 100, 150 mg/kg for 14 consecutive days.	10 female CD-1 (ICR) mice per group	**Ti liver content:** dose-dependent increase. **Interaction with DNA:** TiO_2 was bound on DNA, caused changes in DNA conformation, induced DNA cleavage.

Table 5: Continued.

References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
		Liver		
Tang et al., 2010 [75]	TiO_2 (5)	**Intratracheal instillation:** 0.8, 4, 20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**Biochemical parameters:** ALT increased. **^{1}H NMR urine analysis:** increase in valine, lactate,acetate, succinate, 2-OG, creatinine, taurine, TMAO, allantoin, hippurate1-2; decrease in citrate, DMA.
Tang et al., 2011 [40]	Anatase TiO_2 (5 ± 1)	**Intratracheal instillation:** 0.8, 4, 20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**Biochemical parameters:** ALB and GLU increased. **^{1}H NMR serum analysis**: ketone bodies, choline, LDL, alanine, and GLU increased; lactate, creatine, and pyruvate decreased. **TEM analysis:** swollen hepatocytes, congested sinusoids.
Nemmar et al., 2011 [79]	Rutile Fe-doped TiO_2 (length: 80; diameter: 7)	**Intratracheal instillation:** 1, 5 mg/kg TiO_2	4 male Wistar rats per group	**Biochemical parameters:** AST and ALT increased. **Histology:** inflammatory cell infiltration, mainly LYMs.
Yamashita et al., 2011 [100]	TiO_2 (35)	**Intravenous injection:** 0.8 mg TiO_2 for 2 consecutive gestational days.	Pregnant mice	**Ti distribution:** TiO_2 detected in fetal liver

2-OG, 2-Oxoglutarate; 3-D-HB, 3-D-hydroxybutyrate; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase activity; ChE, cholinesterase; CRP, cross-reaction protein; CYP1A, cytochrome p450 1A; DMA, Dimethylamine; GLB, globulin; GLU. Glutamic acid; GSH-Px, glutathione peroxidase; GST, glutathione-S-transferase; HDL-C, high-density lipoprotein cholesterol; HSP70, heat shock protein 70; IL-, interleukin; LDL-C, low-density lipoprotein cholesterol; LAP, leucine acid peptide; LDH, lactate dehydrogenase; LYM, lymphocyte; MDA, malondialdehyde; MIF, migration inhibitory factor; MT, metallothionein; NO, nitric oxide; NEU, neutrophil; NF-kB, nucleic factor-kB; NIK, NF-κB-inducible kinase; NMR, nuclear magnetic resonance; PAG, phenylacetylglycine; PChE, pseudocolinesterase; SOD, superoxide dismutase; TBIL, total bilirubin; TCHO, total cholesterol; TEM, transmission electron microscopy; TF, transferrin; TG, triglycerides; TLR-, toll-like receptor-; TMAO, trimethylamine-N-oxide; TiO_2 NPs, titanium dioxide nanoparticles; TNF-α, tumor necrosis factor-α; TP, total protein.

Resonance (NMR) spectra of urine and serum [40, 75, 129], both after repeated intragastric doses of a mixture of rutile and anatase TiO_2 NPs [129] and after acute intratracheal instillation of no characterised TiO_2 NPs [75]. These findings support the role of TiO_2 NPs in leading disturbances in the energy and amino acids metabolism through hepatotoxic effects and confirm the changes in liver function previously described.

Regarding the intravenous exposure, Yamashita et al. [100] detected TiO_2 NPs in fetal liver after NP injections into pregnant mice, Fabian et al. [138] failed to show any hepatic toxicity in rats treated with injection of a mixture of anatase and rutile TiO_2 NPs.

Negative results in terms of alteration in liver parameters were reported also in rats treated with a single intratracheal instillation of TiO_2 NPs [30]. Interestingly, in this study, even if functional and histological liver lesions could not be demonstrated, hepatic superoxide dismutase (SOD) activity was reduced, and MDA levels were increased suggesting a possible role of oxidative stress and lipid peroxidation generated by NP exposure, perhaps as an early sign of damage, previous to clear functional parameter alterations. These findings are in line with the *in vivo* results previously detailed [115, 135, 136] and those obtained *in vitro* by Shi et al. [139] showing increased ROS and MDA in TiO_2 NP-exposed hepatocytes.

In conclusion, though all of the studies reviewed in this section concentrate on hepatotoxic effects of TiO_2 NPs, the current knowledge in this field is not yet exhaustive, and further investigation is necessary to fully elucidate the pathogenesis of the liver damage and the potential relationship between liver toxicity and the different characteristics of NPs. Interestingly, the interactions between TiO_2 NPs and DNA, both direct or indirect, such as those mediated by oxidative stress, deserve greater attention in order to the understand their potential role in the mechanisms underlining genotoxic and carcinogenic effects. Moreover, no data are present in literature on the *in vivo* effects of NPs on the gastric and intestinal systems, and only limited studies on cells of the latter system have been performed *in vitro*. In our opinion, this aspect should be deeply investigated in future research due to its great importance, particularly considering the possibility of NPs penetrating into organisms through the intestines.

2.6. Hematopoietic and Immunological Systems. Only few studies have investigated the influence of TiO_2 NP exposure on hematological parameters reporting conflicting results (Table 6). Evidence for NP-related upregulation of systemic inflammation, assessed by increased white cell count, was provided by several studies [43, 79, 129], whereas Duan et al. [21] failed to confirm these findings. Particularly, they demonstrated that the reduced levels of CD3, CD4, CD8-competent T lymphocytes, B, and natural killer (NK) cells and the reduced ratio of CD4 to CD8 detected were a clear sign of a disturbance of the cellular immune function and of the inhibition of the immune response of mice. Differences in the TiO_2 form used, rutile [43, 79] versus anatase [21] versus a mixture of the two [129], the shape of the particles, nanorods [43, 79] versus NPs [21, 129], administration route, intratracheal [43, 79] versus intragastric [21, 129], and the length of exposure, acute [43, 79] versus subchronic [21, 129] may account for the discrepancies between the previous reviewed studies.

Moreover, Nemmar et al. [43] detected a reduced number of platelets *in vivo* due to a possible aggregation confirming the results obtained by the same authors *in vitro*. Unfortunately, these results were not confirmed when rats were intratracheally exposed to Fe-doped TiO_2 nanorods, suggesting a potential role of the particle surface chemistry in influencing hematological effects *in vivo* [79].

However, all of these data should be interpreted as preliminary results and must be confirmed by future studies.

Other recent works have also investigated the effects of anatase TiO_2 NPs on a key organ of the immunological system, the spleen, following both intraperitoneal injections [22, 116, 122, 140] or intragastric administrations [141] (Table 6). These studies revealed that TiO_2 NPs are able to accumulate in the spleen in a dose-dependent manner [22, 122, 140] inducing histopathological damage. Apoptotic morphological changes [140], accumulation of neutrophils [22], congestion of the splenic tissue, and lymph node proliferation [140, 141] were detected. Moreover, reduction in total splenocyte, CD4 and CD8 T-lymphocyte number, retardation in B-lymphocyte development, and reduction in LPS stimulated NK cells were reported after exposure to TiO_2 NPs [116].

A recent study [71], demonstrated that, in ovalbumin-sensitized mice, silica, coated rutile TiO_2 NP inhalation decreased TNF-α and IL-13 expression in spleen cells.

On the other hand, Wang et al. [121] failed to reveal abnormal pathological changes in mouse spleen after a single intragastric administration. Unfortunately, this study lacks details regarding the form of the TiO_2 employed.

TiO_2 NPs induced increased ROS and MDA in the spleen [140, 141] and decreased levels of SOD antioxidant activity in plasma after intratracheal instillation in rats [30]. Oxidative stress was thought to play an important role in triggering the mitochondrial-mediated apoptotic pathway demonstrated in the spleen tissue [140].

However, the limited number of studies investigating the hematological and immunological effects of TiO_2 NPs do not allow comparison and extrapolation of certain conclusions. Further research is necessary to confirm these results and to shed light on the roles of NP characteristics in inducing the alterations described above.

2.7. Renal System. Several studies report alterations, although opposite in some cases, of renal functional parameters, such as increased [40, 75, 121, 131] and reduced [122, 134, 142] blood urea nitrogen (BUN), increased [75, 121, 142] and decreased [134] creatinine (Cr), and reduced uric acid (UA) levels [122, 142] (Table 7). The impact of TiO_2 NP treatment on the renal function was ulteriourly confirmed by the altered levels of metabolic products in the urine and serum of intratracheally exposed rats

Table 6: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on hematopoietic and immunological systems.

Hematopoietic and immunological systems				
References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
Wang et al., 2007 [121]	TiO_2 (25, 80, 155)	**Intragastric administration**: 5 g/kg TiO_2 in a minute.	20 male and female CD-1 (ICR) mice per group	**Spleen histology:** no alterations.
Nemmar et al., 2008 [43]	Rutile TiO_2 nanorods (4–6)	**Intratracheal instillation**: 1, 5 mg/kg TiO_2.	6-7 male Wistar rats per group	**Blood parameters:** increased MONs and GRAs, decreased PLTs.
Chen et al., 2009 [22]	Anatase TiO_2 (80–110)	**Intraperitoneal injections**: 324–2592 mg/kg TiO_2.	10 male and female ICR mice.	**Spleen histology:** severe lesions; NEU infiltration.
Li et al., 2010 [140]	Anatase TiO_2 (~6-7)	**Intraperitoneal injections**: 5–150 mg/kg TiO_2 every day for 45 days.	20 female CD-1 (ICR) mice per group	**Oxidative stress in spleen:** increased ROS and MDA. **Spleen histology:** congestion, lymph nodule proliferation, splenocyte apoptosis. **Apoptosis mechanism:** TiO_2 activated caspase −3 and −9, decreased Bcl-2, increased Bax and cytochromec.
Liang et al., 2009 [30]	TiO_2 (5, 21)	**Intratracheal instillation**: 0.5–50 mg/kg TiO_2.	6 male and female Sprague Dawley rats per group	**Oxidative stress:** decreased SOD activity in plasma.
Duan et al., 2010 [21]	Anatase TiO_2 (5)	**Intragastric administration**: 62.5–250 mg/kg TiO_2	20 female CD-1 (ICR) mice per group	**Blood parameters:** WBC, RBC, Hb, mean corpuscular Hb concentration, thrombocytes, reticulocytes decreased; mean corpuscular volume, mean corpuscular Hb, red cell distribution width, PLTs, HT, mean PLT volume increased. **Immunological parameters:** CD3, CD4, CD8, CD4/CD8, B, and NK cells decreased. **Inflammatory action:** IL-2 decreased and NO increased by TiO_2.
Bu et al., 2010 [129]	Rutile-anatase TiO_2 mixture (<50)	**Intragastric administration**: 0.16–1 g/kg TiO_2 once a day for 14 consecutive days.	16 male and female Wistar rats per group	**Blood parameters:** WBC, LYMs, MONs, EOS increased. **Spleen histology:** no alterations.
Rossi et al., 2010 [71]	Silica coated rutile TiO_2 (~10 × 40) Rutile TiO_2 (<5 μm)	**Inhalation**: 10 ± 2 mg/m^3 TiO_2 for 2 hr a day, 3 days a wk, for 4 wks.	8 female BALB/c/Sca mice per group	**Inflammatory action:** TiO_2 NPs decreased TNF-α and IL-13 expression in spleen cells.
Nemmar et al., 2011 [79]	Rutile Fe-doped nanorod TiO_2 (length: 80; diameter: 7)	**Intratracheal instillation:** 1, 5 mg/kg TiO_2	4 male Wistar rats per group	**Blood parameters:** WBC, IL-6, SOD, GSH, PLTs increased.
Moon et al., 2011 [116]	TiO_2 (<25, <100)	**Intraperitoneal injections**: once a day for 7 days	Mice	**Spleen cells:** splenocytes, CD4+, LPS stimulated NK cells CD8+ decreased; B-lymphocyte development and LPS-stimulated spleen cell proliferation were retarded by TiO_2.

Table 6: Continued.

References	Crystal phase composition (particle size in nm)	Hematopoietic and immunological systems Type of exposure	Type and number of animals	Results
Wang et al., 2011 [141]	Anatase TiO_2	**Intragastric administration**: 5–150 mg/kg TiO_2 for 30 consecutive days.	20 female CD-1 (ICR) mice per group	**Spleen histology:** congestion, lymph nodule proliferation. **Oxidative stress:** O_2^-, H_2O_2, MDA levels, and p38, JNK, NF-kB, Nrf-2, and HO-1 expression increased in spleen.

EOS, eosinophil; GRA, granulocyte; GSH, reduced glutathione; Hb; haemoglobin; HT, hematocrit; HO-1, Heme oxygenase-1; IL-, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LYM, lymphocyte; MDA, malondialdehyde; MON, Monocyte; NEU, neutrophil; NF-kB, nucleic factor-kB; NK, natural killer; Nrf-2, nuclear factor-E2-related factor-2; PLT, platelet; RBC, red blood cells; ROS, reactive oxygen species; SOD, superoxide dismutase; TiO_2 NPs, titanium dioxide nanoparticles; TNF-α, tumor necrosis factor-α; WBC, white blood cells.

Table 7: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on renal system.

References	Crystal phase composition (particle size in nm)	Renal system Type of exposure	Type and number of animals	Results
Wang et al., 2007 [121]	TiO_2 (25, 80, 155)	**Intragastric administration**: 5 g/kg TiO_2 in a minute.	20 male and female CD-1 (ICR) mice per group	**Biochemical parameters:** BUN, Cr increased by 25, 80 nm TiO_2. **Histology:** proteinic liquids in renal tubules, glomerulus swelling.
Fabian et al., 2008 [138]	Anatase-rutile TiO_2 mixture (20–30)	**Intravenous injection:** 5 mg/kg	3 male Wistar rats per group	**Biochemical parameters:** no alterations in BUN and Cr.
Liang et al., 2009 [30]	TiO_2 (5, 21)	**Intratracheal instillation**: 0.5–50 mg/kg TiO_2.	6 male and female Sprague Dawley rats per group	**Biochemical parameters:** no alterations in BUN and Cr. **Oxidative stress:** decreased SOD and GSH-PX, increased MDA renal activity (5 nm TiO_2).
Chen et al., 2009 [22]	Anatase TiO_2 (80–110)	**Intraperitoneal injections**: 324–2592 mg/kg TiO_2.	10 male and female ICR mice.	**Biochemical parameters:** no alterations in BUN. **Histology:** glomerulus swelling, proteinic liquids in renal tubules.
Liu et al., 2009 [122]	Anatase TiO_2 (5) Bulk rutile TiO_2 (10–15 μm)	**Intra-abdominal injections**: 5–150 mg/kg BW anatase TiO_2 and 150 mg/kg bulk TiO_2 everyday for 14 days.	10 female CD-1 (ICR) mice per group	**Biochemical parameters**: UA and BUN dcreased by both TiO_2.
Guo et al., 2009 [131]	N.A.	**Intraperitoneal injections**: 200 and 500 mg/kg TiO_2 every other day for 5 times.	15 male ICR mice per group	**Biochemical parameters**: BUN increased.
Wang et al., 2009 [134]	Anatase TiO_2 (diameter: 45.87 ± 7.75; thickness: 10–15)	**Intraarticular injection**: 0.2–20 mg/kg TiO_2 in the knee joints every other day for 4 times.	10 male Sprague Dawley rats per group	**Biochemical parameters:** BUN and Cr decreased. **Histology:** proteinic liquids in renal tubules.
Tang et al., 2010 [75]	TiO_2 (5)	**Intratracheal instillation:** 0.8–20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**Biochemical parameters:** BUN and Cr increased. ^{1}**H NMR urine analysis:** increase in valine, lactate,acetate, succinate, 2-OG, creatinine, taurine, TMAO, allantoin, hippurate1-2; decrease in citrate, DMA.
Tang et al., 2011 [40]	Anatase TiO_2 (5 ± 1)	**Intratracheal instillation:** 0.8–20 mg/kg TiO_2.	8 male Sprague Dawley rats per group	**Biochemical parameters:** BUN increased. ^{1}**H NMR serum analysis:** ketone, bodies, choline, LDL, alanine and GLU increased; lactate, creatine, and pyruvate decreased. **TEM analysis**: tubule epithelial cell damage, vascular deformity.

Table 7: Continued.

References	Crystal phase composition (particle size in nm)	Renal system Type of exposure	Type and number of animals	Results
Zhao et al., 2010 [142]	Anatase TiO_2	**Intra-abdominal injections:** N.A.	Mice	**Biochemical parameters:** Cr, Ca^{2+}, phosphonium increased, BUN and UA decreased. **Oxidative stress:** ROS, LPO increased, superoxide dismutase, catalase, ascorbate peroxidase, total antioxidant capacity, glutathione, and ascorbic acid content decreased.

2-OG, 2-Oxoglutarate; BUN, blood urea nitrogen; Cr, creatinine; DMA, Dimethylamine; GLU, glutamic acid; GSH-Px, glutathione peroxidase; LDL, low-density lipoprotein; LPO, lipid peroxidation; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TiO_2 NPs, titanium dioxide nanoparticles; TMAO, trimethylamine-N-oxide; UA, uric acid.

investigated by ^{1}H NMR analysis [40, 75]. However, the clear interpretation of these data and the causative mechanisms are not fully understood. Unfortunately, of these studies, only few characterize the TiO_2 form used as anatase [40, 122, 134, 142], while the others lack TiO_2 particle characterization [75, 121, 131]. Moreover, the different routes of exposure employed to investigate nephrotoxicity, such as intraperitoneal [122, 131, 142], intra-articular [134], intragastric [121], and intratracheal [75] administrations, do not allow a clear comparison of the results obtained.

On the other hand, different studies did not report TiO_2 NP-induced nephrotoxicity neither following intravenous exposure to a mixture of anatase and rutile [138] nor after anatase intraperitoneal injections [22], nor following intratracheal instillation with no characterized TiO_2 NP suspension [30]. In this latter study, pathological changes were not observed in kidney tissues. In contrast, renal glomerulus swelling [22] and deposition of proteinic liquid in renal proximal tubules were described [22, 121, 134], also without functional damage [22].

The role of oxidative stress as a possible triggering mechanism for TiO_2 NP inducing kidney damage was demonstrated by increased ROS generation [142], enhanced lipid peroxidation [30], as well as decreased SOD, catalase, ascorbate peroxidase, and glutathione peroxidase (GSH-Px) antioxidant activities [30, 142]. Surprisingly, these changes were detected also in a study where functional alterations were not present, suggesting their role as an early sign of damage, previous to clear functional parameter alterations [30].

These fragmented data require confirmation and further investigation, which should focus on the role played by the different characteristics of TiO_2 NPs in inducing such alterations.

2.8. Musculoskeletal System. Two studies investigated the biological tolerance of TiO_2 NPs when implanted in rat living tissues [143, 144] (Table 8). Interest in this type of research emerges from the promising utilization of NPs in numerous fields of medicine and the not fully understood potential reactions at the tissue-material interface. Inflammation and granulomas were detected in the vicinity of intramuscularly implanted TiO_2 NPs as a normal reaction to a "foreign body," but no signs of intolerance were observed in both studies at 6 or 12 months postimplantation.

Moreover, an inflammatory reaction, characterized by hypertrophy of the sinovial membrane, infiltration of lymphocytes and plasma cells, and proliferation of fibroblasts, was observed in rat knee joints when TiO_2 NP suspensions were intraarticularly injected [134]. NPs were also able to stimulate oxidative stress as demonstrated by increases of antioxidants, such as GSH-Px and SOD activities, which were elevated to overcome an increase in ROS production.

TiO_2 NPs were able to convert benign nonmetastatic tumor cells into malignant metastatic ones in a manner dependent on the NP surface chemistry and the ability to produce ROS in the target cells [145]. In particular, poorly tumorigenic and nonmetastatic QR-32 fibrosarcoma cells became tumorigenic only after injection into subcutaneous sites previously implanted with hydrophilic $ZrO_2Al(OH)_3$-treated TiO_2 NPs. Moreover, tumor cell lines derived from the QR-32 cells of both TiO_2 NP preimplanted sites acquired a pulmonary and extrapulmonary metastatic phenotype when intravenously injected in mice. Both the 8-hydroxy-2′-deoxyguanosine (8-OHdG) adduct and 4-hydroxy-2-nonenal-positive cells, induced by oxidative stress action, were increased in the hydrophobic $ZrO_2Al(OH)_3$ plus steric acid-treated TiO_2 NP implantation sites compared to the hydrophilic one.

In conclusion, future research should investigate musculoskeletal tissue-TiO_2 NP interactions, especially considering the potential widespread utilization of TiO_2 NPs in biomedical products.

2.9. Reproductive System. Only few studies investigated the effects of TiO_2 NPs on the male reproductive system [101, 131] (Table 8). Guo et al. [131] demonstrated reductions in sperm density and motility and an increase in sperm abnormality and germ cell apoptosis in mice treated with intraperitoneal injections of TiO_2 NPs. Testes and epididymis did not show pathological changes.

When mice were prenatally exposed to anatase TiO_2 NPs via subcutaneous injections of dams they showed aggregates of NPs in Leydig cells, Sertoli cells, and spermatids in the testes [101]. Disorganized and disrupted seminiferous tubules, tubule lumens with few mature sperm, as well as decreases in daily sperm production, epididymal sperm motility, and numbers of Sertoli cells were also observed [101, 146].

Such limited knowledge regarding the reproductive toxicological effects of TiO_2 NPs and the relevance of this topic makes future investigations a matter of urgency.

2.10. Genotoxicity. TiO_2 NP genotoxicity is poorly studied *in vivo*, and the mechanisms involved have not been clearly defined. To our knowledge, only two studies have investigated *in vivo* genotoxic effects and report contrasting results (Table 9). One study [147] demonstrated that TiO_2 NPs are inert to lung cells, while the other [148] found that NPs induce genotoxicity in organs such as the blood, bone marrow, and embryos.

Specifically, Rehn et al. [147] showed that both hydrophilic P25 Degussa TiO_2 NPs and hydrophobic trimethoxy-octylsilane-treated T805 TiO_2 NPs intratracheally instilled in rats failed to induce persistent inflammation in BAL. The single lung cell immunohistochemical detection of 8-oxoguanine revealed no differences between both NP-treated groups and controls.

Trouiller et al. [148] exposed adult and pregnant dam mice to water supplemented with solutions containing P25 Degussa TiO_2 NPs. Eyespots, a measure of DNA deletion, increased in *in utero* TiO_2 NP-treated mice compared to controls. A dose-dependent increase in DNA double-strand breaks was also detected in bone marrow cells and an increased tail moment was evident in peripheral white blood cells of treated mice. MN frequency and the level of 8-OHdG were higher in peripheral blood erythrocytes and in

Table 8: *In vivo* studies that investigated the adverse effects of TiO_2 NPs on musculoskeletal and reproductive systems.

References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
		Musculoskeletal system		
Hansen et al., 2006 [143]	TiO_2 (70) Bulk TiO_2 (diameter: 0.9 mm; height: 1 mm)	**Subcutaneous (bulk) and intramuscular (NPs) implantation** in rat back for 6 or 12 months.	10 Male Sprague Dawley rats	**Local reaction:** presence of granulomas (NPs), inflammatory infiltrates (bulk); no local intolerance (both).
Gatti et al., 2008 [144]	TiO_2 (70) Bulk TiO_2 (diameter: 6–12 mm; height: 2 mm)	**Subcutaneous (bulk) and intramuscular (NPs) implantation** in rat back for 6 or 12 months.	10 male Sprague Dawley rats	**Local reaction:** granulomas (NPs), inflammatory infiltrates (bulk).
Wang et al., 2009 [134]	Anatase TiO_2 (diameter: 45.87 ± 7.75; thickness: 10–15)	**Intraarticular injection**: 0.2, 2, 20 mg/kg TiO_2 in the knee joints every other day for 4 times.	10 male Sprague Dawley rats per group	**Oxidative stress in synovium:** GSH-Px, GSH, GSSG, MDA, and SOD increased. **Histology:** synovium hypertrophy, LYMs, plasma cell infiltration, and fibroblast proliferation.
Onuma et al., 2009 [145]	Hydrophilic rutile TiO_2 treated with $ZrO_2Al(OH)_3$ (minor axis: 40–70; major axis 200–300) Hydrophobic rutile TiO_2 treated with $ZrO_2Al(OH)_3$ and steric acid (minor axis: 40–70; major axis 200–300)	**Subcutaneous injections**: QR-32 cells alone; QR-32 cells mixed with 5 mg/0.1 mL TiO_2; and 5 mg/0.1 mL TiO_2 30 and 70 days before QR-32 cells injection. **Intravenous injections**: tumor cell lines into mice (5/line).	15 female C57BL/6 mice per group	**Carcinogenicity:** QR-32 cells became tumorigenic after injection in sites implanted for 30 days or 70 days with hydrophilic TiO_2. **Metastatic ability:** acquired by tumor cell lines derived from QR-32 cells injected in site preimplanted with both TiO_2.
		Reproductive system		
Guo et al., 2009 [131]	N.A.	**Intraperitoneal injections**: 200 and 500 mg/kg TiO_2 every other day for 5 times.	15 male ICR mice per group	**Reproductive effects**: reduced sperm density and motility, increased sperm abnormality, and germ cell apoptosis.
Takeda et al., 2009 [101]	Anatase TiO_2 (25–70)	**Subcutaneous injections**: 100 μL of TiO_2 at 1 mg/mL at 3, 7, 10 and 14 days post-coitum.	6 pregnant Slc:ICR mice per group	**Reproductive effects:** disorganised and disrupted seminiferous tubules, few mature sperm, decreased sperm production, epididymal sperm motility, number of Sertoli cells.

GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; LYM, lymphocyte; MDA, malondialdehyde; SOD, superoxide dismutase; TiO_2 NPs, titanium dioxide nanoparticles.

Table 9: *In vivo* studies that investigated the genotoxic effects of TiO_2 NPs.

		Genotoxicity		
References	Crystal phase composition (particle size in nm)	Type of exposure	Type and number of animals	Results
Rehn et al., 2003 [147]	P25 Degussa TiO_2 (~20) T805 TiO_2 (~20)	**Intratracheal instillation**: 0.15–1.2 mg TiO_2.	30 female Wistar rats per group	**Inflammatory action:** TCC, AMs, NEUs, TPC, phosphatidylcholine, TNF-α increased. **Genotoxicity:** no changes in 8-oxoGua levels.
Trouiller et al., 2009 [148]	P25 Degussa TiO_2 (21)	**Oral administration**: 5 mL of 60–600 μg/mL TiO_2 for 5 consecutive days. Pregnant dams: supplemented TiO_2 drinking water for 10 days at a concentration of 300 μg/mL.	5 C57Bl/6Jp^{un}/p^{un} mice per group	**Inflammatory action:** blood TNF-α, IFN-γ, KC increased. **Genotoxicity:** increase in DNA deletion in *in utero* treated mice (eyespots per RPE); DNA double strand breaks in bone marrow cells (γ-H2AX foci formation) and in WBC (comet assay); MN in ERYs; 8-OhdG in liver.

8-OhdG, 8-hydroxy-2′-deoxyguanosine; 8-oxoGua, 8-oxoguanine; AM, alveolar macrophage; ERY, erythrocyte; IFN-γ, interferon- γ; KC, mouse orthologue of interleukin-8; MN, micronuclei; NEU, neutrophil; RPE, retinal pigment epithelium; TCC, total cell count; TiO_2 NPs, titanium dioxide nanoparticles; TNF-α, tumor necrosis factor-α; TPC, total protein content; WBC, white blood cells.

the livers of TiO_2 NP-exposed mice, respectively. Moreover, TNF-α, interferon (INF)-γ and the mouse orthologue of IL-8 were increased after exposure. These studies evaluated both genotoxic effects and inflammatory parameters, because most *in vitro* TiO_2 NP genotoxicity studies have suggested that NPs could damage DNA directly or indirectly via oxidative stress and/or inflammatory responses [148]. The discrepancies between *in vivo* results reproduce the conflicting *in vitro* findings [33]. However, though the limited number of *in vivo* studies does not allow one to extrapolate conclusions, we hypothesize that different TiO_2 forms, particle sizes, degrees of aggregation, preparation methods, incubation conditions, doses, and susceptibility between cell types influence responses to TiO_2 NPs.

Interestingly, the study carried out by Trouiller et al. [148] is the first showing that DNA deletions in offspring are increased by *in utero* exposure to TiO_2 NPs. This finding is of great importance considering the high susceptibility of embryonic cells to DNA damage and widespread environmental and occupational TiO_2 NP exposure.

In conclusion, there are concerns regarding the potential risk of genetic disorders, particularly for people occupationally exposed to high doses of TiO_2 NPs. Thus, further research is necessary to fully understand genotoxic TiO_2 NP effects and to determine the conditions in which they occur to fully evaluate NP exposure risks.

3. Discussion

Our analysis pointed out interesting and critical aspects, particularly in relation to the interaction between NP-induced effects and remarkable treatment parameters, such as routes of exposure, TiO_2 form, NP size, and surface reactivity, that we argue will be the focus of future research. In all the studies reviewed, the commonly proposed pathogenic mechanisms initiated by TiO_2 NPs are dominated by inflammation-driven effects, including fibrosis, oxidative stress, and DNA damage, making inflammation a target for toxicological testing [7, 11, 149, 150]. Regarding the respiratory tract, which is considered the most affected organ, lung overload is thought to be responsible for the induction of inflammation, ROS production, and ultimately lung damage. Indeed, it should be carefully evaluated in future studies for both its "volumetric," "mass," and "surface area" determinants. Future research will be also necessary to clarify which NP characteristic could play a triggering role in the cascade of events responsible for lung injury. Moreover, a correct evaluation of the risk derived from TiO_2 NP respiratory exposure should be performed in consideration of the significant role of this route of exposure for both the general and occupational populations. Particularly, in the latter setting, an appropriate risk assessment focused on the pattern of absorption, the role of the total inhaled amount, direct lung alteration, and/or damages in other distant organs, could greatly influence both the risk communication and the risk management phases, the adoption of valuable methods of environmental and biological monitoring, and the measures of collective and individual protection.

Interestingly, inhaled TiO_2 NPs resulted able to be translocate to the CNS via the axons of sensory neurons in the upper respiratory tract [31, 91, 92]. This is an intriguing aspect because it introduces a route for TiO_2 NP exposure directly linked to the CNS, in which NPs could elicit their toxic effects. These findings underscore the need for additional studies to further elucidate underlying mechanisms and to characterize the physiological impact, particularly in relation to which NP characteristics could influence this passage. The confirmation of this potential route of exposure may be extremely important for the general and occupational population, because the direct translocation of NPs to the CNS through the olfactory bulb could requires greater attention in public and workplace settings, as well as the urgent adoption of adequate protective measures.

Dermal studies have shown that there is currently little evidence for skin penetration and systemic exposure after dermal applications of TiO_2 NPs used in sunscreens [107, 109, 112, 113]. However, the open question has been raised as to whether the usual testing with healthy, intact skin is sufficient [151, 152].

Regarding other organs, the data are too limited to extrapolate general concepts, and additional studies are required, which may offer new details regarding TiO_2 NP absorption, systemic uptake, and kinetics, providing a deeper knowledge of the TiO_2 NP toxicological profile. In this context, it could be helpful to investigate new, noninvasive, powerful techniques aimed to identify early pathological metabolic and toxicological processes induced by NPs [40, 153–155]. Among these methods, metabonomics provide a relatively complete biological summary of the whole body response to NPs, and an appropriate identification of tissue damage biomarkers [40].

Moreover, while TiO_2 has been classified as possibly carcinogenic to humans (Group 2B) [28, 29], no definite conclusion is present for TiO_2 NPs. The NIOSH recently determined that inhaled ultrafine TiO_2 is a potential occupational carcinogen and recommended an exposure limit of 0.3 mg/m^3 for ultrafine (including engineered nanoscale) TiO_2 as a TWA concentration for up to 10 hr/day during a 40-hour work week [15]. This conclusion was due to weight of scientific data demonstrating TiO_2 NP carcinogenic effect on rat lungs [56]. Interestingly, NIOSH has reviewed that the tumor response observed in rats exposed to TiO_2 NPs resulted from a secondary genotoxic mechanism involving chronic inflammation and cell proliferation [15]. The central hypothesis is that inflammation leads genotoxic events as well as cell proliferation and tissue remodeling, which are processes that are all required for mutations and progression towards neoplastic lesions [156]. However, the mutagenic or epigenetic mechanisms underlying the TiO_2 NP carcinogenic action need ulterior confirms and their role in the multistep process of carcinogenesis, likely as trigger of the initiation, promotion or progression phases of tumor manifestation, should be deeply elucidated [33]. This argument merits much attention in future studies with a particular focus on the conditions in which TiO_2 NP genotoxicity and carcinogenicity occur particularly in relation to the potential influence played by different particle production, size, degree

of aggregation, preparation method, exposure conditions, dose, and susceptibility of animal species [33, 148, 157, 158]. These results will be of great importance in order to deeply characterize the risk of TiO_2 NP exposure and to define suitable exposure limits.

The relevance of the toxicological research in animals lies on the possibility to evaluate the type and entity of the adverse response induced by different doses of TiO_2 NPs and to extrapolate threshold levels aimed to protect exposed human populations. The general dose-response model indicates that the higher the concentration of a chemical substance, the greater is the influence on the organism [5]. However, in nanotoxicological research, the dose-response relationship requires special attention considering that the traditional mass dose does not well reflect the biologically effective dose for NPs. Other dose metrics, such as surface area combined with surface reactivity or the particle number, should be evaluated as ulterior, maybe better, descriptors of the potential to cause damage at the site of particle deposition or far from this [32, 82, 159, 160]. In this context, a TiO_2 NP standardized characterization including parameters like TiO_2 form, NP number, and mass concentration, size, shape, state of agglomeration, charge, crystallinity, solubility, oxidant generation capacity, added functional groups, or impurities and rate of dissolution is the first step to a comprehensive identification of the TiO_2 NP hazard [2, 7]. It seems necessary to focus future research on establishing a matrix of relationship between specific NP properties and resultant biological activities. In fact, nanotoxicology literature indicates that the combination of various physicochemical features in any type of NP leads to different interaction with biological systems and consequently to different toxic potentials [34, 61, 161, 162]. A deeper understanding of parameters able to alter the TiO_2 NP bioactivity would support a practical approach in the development of "safe" engineered NPs, through the choice of a particular TiO_2 form, shape, or surface functionalization [163].

From the studies, we reviewed a great methodological heterogeneity has emerged in terms of type of TiO_2 NPs employed, animal species treated, route of exposure, doses applied, endpoint parameters, and techniques of measurement. This causes a biased assessment of the exposure-response relationships, prevents comparison between results and extrapolation of definite conclusions [161]. Moreover, results obtained employing extremely high doses of TiO_2 NPs and unrealistic routes of administration, such as bolus instillation or aspiration delivery, should be interpreted with caution as preliminary findings to be validated under more realistic exposure conditions [164]. However, these preliminary data could be useful to plan future experimental projects aimed to reveal early signs of more severe damages induced by higher dose treatments [33, 165].

Currently, concerns regarding nanotechnology in everyday life are emerging, particularly those relative to the risks posed by occupational exposure, in consideration of the increasing number of workers employed in research, manufacture, use, and disposal of nanomaterials [163, 166]. Indeed, although not definite, the potential risk for adverse effects in NP exposed workers demands that industry, labor, and government concert action to a precautionary NP risk management to protect worker health [161, 167–172]. Critical steps in reaching this target are an appropriate risk assessment, based on epidemiologic research and exposure evaluation, and the determination of the effective function of risk management programs [163]. Epidemiologic studies are essential in understanding health outcomes associated with exposure to potentially hazardous materials. Such studies will form the basis for quantitative risk estimations to establish levels that protect human health. Currently, only NIOSH has recommended a TiO_2 NP exposure limit, however, regulating TiO_2 NPs appears to be a challenging task due to the broad diversity of the NP characteristics and their application in a wide range of sectors and products [173]. Moreover, it has to be questioned if a generic threshold limit, without any specification relative to the particle characterization and the context of employment, could have a suitable role in protecting health and safety of occupational exposed subjects.

NP exposure in workplace settings should receive much attention in future research. At present, the relative newness of exposure scenarios, generally occurring in controlled situations, the inconsistencies over the way to identify and classify nanomaterials, questions about metrics and practical instrumentation, and difficulty in gaining access to workplaces mostly prevent such essential assessment [174–180].

Additionally, as a part of the occupational exposure and health effect evaluation, future studies should focus at identifying NP-associated biomarkers. In this regard, a deeper knowledge about TiO_2 NP mechanisms of disease, and TiO_2 NP biokinetics and potential health effects, obtained from *in vitro* and *in vivo* experiments, respectively, could be useful to identify indicators of early biological events and to establish appropriate methods for their detection. Experimentally identified biomarkers should be the focus of subsequent validation studies aimed to define their ability in exploring human disease endpoints [6]. Such confirmed biological indicators of exposure and effect could be of great relevance to the development of occupational health surveillance strategies and also to verify the appropriateness of collective and individual defense systems adopted during NP manipulation.

Finally, interpreting and communicating hazard and risk information is an integral part of the TiO_2 NP risk management that clearly requires additional research to more thoroughly understand the toxicological interactions of these NPs with biological systems at the molecular, cellular, organ, and whole body level.

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