Prasad RY, Wallace K, Daniel KM, Tennant AH, Zucker RM, Strickland J, Dreher K, Kligerman AD, Blackman CF and DeMarini DM, 2013. Effect of treatment media on the agglomeration of titanium dioxide nanoparticles: impact on genotoxicity, cellular interaction, and cell cycle. ACSNano, 3, 1929–1942.

Proquin H, Rodriguez-Ibarra C, Moonen CG, Urrutia Ortega IM, Briede JJ, de Kok TM, van Loveren H and Chirino YI, 2017. Titanium dioxide food additive (E171) induces ROS formation and genotoxicity: contribution of micro and nano-sized fractions. Mutagenesis, 32, 139–149.

Prüst M, Meijer J and Westerink RHS, 2020. The plastic brain: neurotoxicity of micro- and nanoplastics. Part Fibre Toxicol, 17, 24.

Quintanilla I, López-Cerón M, Jimeno M, Cuatrecasas M, Zabalza M, Moreira L, Alonso V, Rodríguez de Miguel C, Muñoz J, Castellvi-Bel S, Llach J, Castells A, Balaguer F, Camps J and Pellisé M, 2019. Rectal aberrant crypt foci in humans are not surrogate markers for colorectal cancer risk. Clin Transl Gastroenterol, 10, e00047.

Rahman Q, Lohani M, Dopp E, Pemsel H, Jonas L, Weiss DG and Schiffmann D, 2002. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. Environmental Health Perspectives, 110, 797–800.

Rahnama S, Hassanpour A, Yadegari M, Anvari M and Hosseini-sharifabad M, 2020. Effect of titanium dioxide nanoparticles on the stereological parameters of the dentate gyrus and the morphology of granular hippocampal neurons in mice. International Journal of Morphology, 38, 1623–1630.

Ranjan S and Ramalingam C, 2016. Titanium dioxide nanoparticles induce bacterial membrane rupture by reactive oxygen species generation. Environmental Chemistry Letters, 14, 487–494.

Rasmussen K, Mast J, De Temmerman P, Verleysen E, Waegeneers N, Van Steen F, Pizzolon J, De Temmerman L, Van Doren E, Jensen K, Birkedal R, Levin M, Nielsen S, Koponen I, Clausen P, Kofoed-Sørensen V, Kembouche Y, Thieriet N, Spalla O, Giuot C, Rousset D, Witschger O, Bau S, Bianchi B, Motzkus C, Shivachev B, Dimowa L, Nikolova R, Nihtianova D, Tarassov M, Petrov O, Bakardjieva S, Gilliland D, Pianella F, Ceccone G, Spampinato V, Cotogno G, Gibson P, Gaillard C and Mech A, 2014. Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: characterisation and physico-chemical properties. JRC86291. https://doi.org/10.2788/79760

Rehn B, Seiler F, Rehn S, Bruch J and Maier M, 2003. Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: untreated and surface treated. Toxicology and Applied Pharmacology, 189, 84–95.

Relier C, Dubreuil M, Lozano Garcìa O, Cordelli E, Mejia J, Eleuteri P, Robidel F, Loret T, Pacchierotti F, Lucas S, Lacroix G and Trouiller B, 2017. Study of TiO_2 P25 nanoparticles genotoxicity on lung, blood, and liver cells in lung overload and non-overload conditions after repeated respiratory exposure in rats. Toxicological Sciences, 156, 2.

Renz H, Brandtzaeg P and Hornef M, 2012. The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. Nature Reviews-Immunology, 12, 9–23.

Riedle S, Wills JW, Miniter M, Otter DE, Singh H, Brown AP, Micklethwaite S, Rees P, Jugdaohsingh R, Roy NC, Hewitt RE and Powell JJ, 2020a. A murine oral-exposure model for nano- and micro-particulates: demonstrating human relevance with food-grade titanium dioxide. Nano-Micro Small, 16, 2000486.

Rizk MZ, Ali SA, Hamed MA, El-Rigal NS, Aly HF and Salah HH, 2017. Toxicity of titanium dioxide nanoparticles: effect of dose and time on biochemical disturbance, oxidative stress and genotoxicity in mice. Biomedicine, Pharmacotherapy Biomedecine, Pharmacotherapie, 90, 466–472.

Rizk MZ, Ali SA, Kadry MO, Fouad GI, Kamel NN, Younis EA and Gouda SM, 2020. C-reactive protein signaling and chromosomal abnormalities in nanotoxicity induced via different doses of TiO_2 (80 nm) BOOST LIVER FUNCTION. Biological Trace Element Research.

Romano-Keeler J and Weitkamp JH, 2015. Maternal influences on fetal microbial colonization and immune development. Pediatric Research, 77, 189–195.

Rompelberg C, Heringa MB, van Donkersgoed G, Drijvers J, Roos A, Westenbrink S, Peters R, van Bemmel G, Brand W and Oomen AG, 2016. Oral intake of added titanium dioxide and its nanofraction from food products, food supplements and toothpaste by the Dutch population. Nanotoxicology, 10, 1404–1414.

Ronald P, Brown RP, Michael D, Delp MD, Stan L, Lindstedt SL, Lorenz R, Rhomberg LR and Beliles RP, 1997. Physiological parameter values for physiologically based pharmacokinetic models. Toxicology and Industrial Health, 13, 407–484.

Saber AT, Jensen KA, Jacobsen NR, Birkedal R, Mikkelsen L, Møller P, Loft S, Wallin H and Vogel U, 2012. Inflammatory and genotoxic effects of nanoparticles designed for inclusion in paints and lacquers. Nanotoxicology, 6, 453–471.

Sadiq R, Bhalli JA, Yan J, Woodruff RS, Pearce MG, Li Y, Mustafa T, Watanabe F, Pack LM, Biris A, Khan QM and Chen T, 2012. Genotoxicity of TiO_2 anatase nanoparticles 383 in B6C3F1 male mice evaluated using Pig-a and flow cytometric micronucleus assays. Mutation Research, 745, 65–72.

Santonastaso M, Mottola F, Colacurci N, Iovine C, Pacifico S, Cammarota M, Cesaroni F and Rocco L, 2019. In vitro genotoxic effects of titanium dioxide nanoparticles (n-TiO_2) in human sperm cells. Molecular Reproduction and Development, 86, 1369–1377.

Santonastaso M, Mottola F, Iovine C, Cesaroni F, Colacurci N and Rocco L, 2020. In vitro effects of titanium dioxide nanoparticles ($TiO_{(2)}$NPs) on cadmium chloride ($CdCl_{(2)}$) genotoxicity in human sperm cells. Nanomaterials, Basel, 10.

Saquib Q, Al-Khedhairy AA, Siddiqui MA, Abou-Tarboush FM, Azam A and Musarrat J, 2012. Titanium dioxide nanoparticles induced cytotoxicity, oxidative stress and DNA damage in human amnion epithelial (WISH) cells. Toxicology In Vitro, 26, 351–361.

Schneider T, Westermann M and Glei M, 2017. In vitro uptake and toxicity studies of metal nanoparticles and metal oxide nanoparticles in human HT29 cells. Archives of Toxicology, 91, 3517–3527.

Schoeffner DJ, 1999. Organ weights and fat volume in rats as a function of strain and age. Journal of Toxicology and Environmental Health Part A, 56, 449–462.

Shwter AN, Abdullah NA, Alshawsh MA, El-Seedi HR, Al-Henhena NA, Khalifa SA and Abdulla MA, 2016. Chemopreventive effect of Phaleria macrocarpa on colorectal cancer aberrant crypt foci *in vivo*. Journal of Ethnopharmacology, 193, 195–206.

Setyawati MI, Khoo PKS, Eng BH, Xiong S, Zhao X, Das GK, Tan TTY, Loo JSC, Leong DT and NG KW, 2013. Cytotoxic and genotoxic characterization of titanium dioxide, gadolinium oxide, and poly(lactic-co-glycolic acid) nanoparticles in human fibroblasts. Journal of Biomedical Materials Research Part A, 101A, 633–640.

Shelby MD and Witt KL, 1995. Comparison of results from mouse bone marrow aberration and micronucleus test. Environmental and Molecular Mutagenesis, 25, 302–313.

Shelby MD, Erexson GL, Hook GJ and Tice RR, 1993. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. Environmental and Molecular Mutagenesis, 21, 160–179.

Shi Y, Zhang JH, Jiang M, Zhu LH, Tan HQ and Lu B, 2010. Synergistic genotoxicity caused by low concentration of titanium dioxide nanoparticles and p, p'-DDT in human hepatocytes. Environmental and Molecular Mutagenesis, 51, 192–204.

Shi Z, Niu Y, Wang Q, Shi L, Guo H, Liu Y, Zhu Y, Liu S, Liu C, Chen X and Zhang R, 2015. Reduction of DNA damage induced by titanium dioxide nanoparticles through Nrf2 in vitro and in vivo. Journal of Hazardous Materials, 298, 310–319.

Shukla RK, Sharma V, Pandey AK, Singh S, Sultana S and Dhawan A, 2011. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicology In Vitro, 25, 231–241.

Shukla RK, Kumar A, Gurbani D, Pandey AK, Singh S and Dhawan A, 2013. TiO_2 nanoparticles induce oxidative DNA damage and apoptosis in human liver cells. Nanotoxicology, 7, 48–60.

Shukla RK, Kumar A, Vallabani NV, Pandey AK and Dhawan A, 2014. Titanium dioxide nanoparticle-induced oxidative stress triggers DNA damage and hepatic injury in mice. Nanomedicine (Lond), 9, 9.

Singh S, Shi T, Duffin R, Albrecht C, van Berlo D, Höhr D, Fubini B, Martra G, Fenoglio I, Borm PJ and Schins RP, 2007. Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO_2: role of the specific surface area and of surface methylation of the particles. Toxicology and Applied Pharmacology, 222, 141–151.

Siskova A, Cervena K, Kral J, Hucl T, Vodicka P and Vymetalkova V, 2020. Colorectal adenomas-genetics and searching for new molecular screening biomarkers. International Journal of Molecular Sciences, 21, 3260.

Sprong C, Bakker M, Niekerk M and Vennemann F, 2015. Exposure assessment of the food additive titanium dioxide (E 171) based on use levels provided by the industry. RIVM Letter report, 2015-0195.

Sramkova M, Kozics K, Masanova V, Uhnakova I, Razga F, Nemethova V, Mazancova P, Kapka-Skrzypczak L, Kruszewski M, Novotova M, Puntes VF and Gabelova A, 2019. Kidney nanotoxicity studied in human renal proximal tubule epithelial cell line TH1. Mutation Research-Genetic Toxicology and Environmental Mutagenesis, 845, 9.

Srivastava RK, Rahman Q, Kashyap MP, Singh AK, Jain G, Jahan S, Lohani M, Llantow M and Pant AB, 2013. Nanotitanium dioxide induces genotoxicity and apoptosis in human lung cancer cell line, A549. Human and Experimental Toxicology, 32, 153–166.

Stoccoro A, Di Bucchianico S, Uboldi C, Coppede F, Ponti J, Placidi C, Blosi M, Ortelli S, Costa AL and Migliore L, 2016. A panel of in vitro tests to evaluate genotoxic and morphological neoplastic transformation potential on Balb/3T3 cells by pristine and remediated titania and zirconia nanoparticles. Mutagenesis, 31, 511–529.

Stoccoro A, Di Bucchianico S, Coppede F, Ponti J, Uboldi C, Blosi M, Delpivo C, Ortelli S, Costa AL and Migliore L, 2017. Multiple endpoints to evaluate pristine and remediated titanium dioxide nanoparticles genotoxicity in lung epithelial A549 cells. Toxicology Letters, 276, 48–61.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS, 2016. Genotoxicity assessment of intravenously injected titanium dioxide nanoparticles in gpt delta transgenic mice. Mutation Research Genetic Toxicology and Environmental Mutagenesis, 802, 30–37.

Suzuki T, Miura N, Hojo R, Yanagiba Y, Suda M, Hasegawa T, Miyagawa M and Wang RS, 2020. Genotoxicity assessment of titanium dioxide nanoparticle accumulation of 90 days in the liver of gpt delta transgenic mice. Genes Environ.

Swidsinski A, Loening-Baucke V and Herber A, 2009a. Mucosal flora in Crohn's disease and ulcerative colitis – an overview. Journal of Physiology and Pharmacology, 60(supplement 6), 61–71.

Swidsinski A, Ung V, Sydora BC, Loening-Baucke V, Doerffel Y, Verstraelen H and Fedorak RN, 2009b. Bacterial overgrowth and inflammation of small intestine after carboxymethylcellulose ingestion in genetically susceptible mice. Inflammatory Bowel Diseases, 15, 359–364.

Sycheva LP, Zhurkova VS, Iurchenkoa VV, Daugel-Dauge NO, Kovalenko MA, Krivtsova EK and Durnev A, 2011. Investigation of genotoxic and cytotoxic effects of micro- and nanosized titanium dioxide in six organs of mice in vivo. Mutation Research, 726, 8–14.

Taboada-López MV, Herbello-Hermelo P, Domínguez-González R, Bermejo-Barrera P and Moreda-Piñeiro A, 2019. Enzymatic hydrolysis as a sample pre-treatment for titanium dioxide nanoparticles assessment in surimi (crab sticks) by single particle ICP-MS. Talanta, 195, 23–32.

Talamini L, Gimondi S, Violatto MB, Fiordaliso F, Pedica F, Tran NL, Sitia G, Aureli F, Raggi A, Nelissen I, Cubadda F, Bigini P and Diomede L, 2019. Repeated administration of the food additive E171 to mice results in accumulation in intestine and liver and promotes an inflammatory status. Nanotoxicology, 13, 1087–1101.

Talbot P, Radziwill-Bienkowska JM, Kamphuis JBJ, Steenkeste K, Bettini S, Robert V, Noordine ML, Mayeur C, Gaultier E, Langella P, Robbe-Masselot C, Houdeau E, Thomas M and Mercier-Bonin M, 2018. Food-grade TiO_2 is trapped by intestinal mucus in vitro but does not impair mucin O-glycosylatioand short-chain fatty acid synthesis in vivo: implications for gut barrier protection. Journal of Nanobiotechnology, 16, 53.

Tassinari R, Cubadda F, Moracci G, Aureli F, D'Amato M, Mauro Valeri M, De Berardis B, Raggi A, Mantovani A, Passeri D, Rossi M and Francesca Maranghi F, 2014. Oral, short-term exposure to titanium dioxide nanoparticles in Sprague-Dawley rat: focus on reproductive and endocrine systems and spleen. Nanotoxicology, 8, 654–662.

Taurozzi JS and Hackley VA, 2012. Preparation of a nanoscale TiO_2 aqueous dispersion for toxicological or environmental testing. National Institute of Standard and Technology Special Publication. 1200-3, 11 pages (June 2012).

Tavares AM, Louro H, Antunes S, Quarre S, Simar S, De Temmerman P-J, Verleysen E, Mast J, Jensen KA, Norppa H, Nesslany F and Silva MJ, 2014. Genotoxicity evaluation of nanosized titanium dioxide, synthetic amorphous silica and multi-walled carbon nanotubes in human lymphocytes. Toxicology In Vitro, 28, 60–69.

Tennant RW, Margolin BH, Shelby MD, Zeiger E, Haseman JK, Spalding J, Caspary W, Resnick M, Stasiewicz S, Anderson B and Minor R, 1987. Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. Science, 236, 933–941.

Terry LJ, Shows EB and Wente SR, 2007. Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. Science, 318, 1412–1416.

Tomankova K, Horakova J, Harvanova M, Malina L, Soukupova J, Hradilova S, Kejlova K, Malohlava J, Licman L, Dvorakova M, Jirova D and Kolarova H, 2015. Reprint of: cytotoxicity, cell uptake and microscopic analysis of titanium dioxide and silver nanoparticles in vitro. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 85, 20–30.

Toyooka T, Amano T and Ibuki Y, 2012. Titanium dioxide particles phosphorylate histone H2AX independent of ROS production. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 742, 84–91.

Trouiller B, Reliene R, Westbrook A, Solaimani P and Schiestl RH, 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Research, 69, 8784–8789.

Turkez H and Geyikoglu F, 2007. An in vitro blood culture for evaluating the genotoxicity of titanium dioxide: the responses of antioxidant enzymes. Toxicology and Industrial Health, 23, 19–23.

Uboldi C, Urban P, Gilliland D, Bajak E, Valsami-Jones E, Ponti J and Rossi F, 2016. Role of the crystalline form of titanium dioxide nanoparticles: Rutile, and not anatase, induces toxic effects in Balb/3T3 mouse fibroblasts. Toxicology In Vitro: An International Journal Published in Association with BIBRA, 31, 137–145.

Urrutia-Ortega IM, Garduno-Balderas LG, Delgado-Buenrostro NL, Freyre-Fonseca V, Flores-Flores JO, Gonzalez-Robles A, Pedraza-Chaverri J, Hernandez-Pando R, Rodriguez-Sosa M, Leon-Cabrera S, Terrazas LI, van Loveren H and Chirino YI, 2016. Food-grade titanium dioxide exposure exacerbates tumor formation in colitis associated cancer model. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 93, 20–31.

Vaknine S and Soreq H, 2020. Central and peripheral anti-inflammatory effects of acetylcholinesterase inhibitors. Neuropharmacology, 168.

Valentin J, 2002. International Commission on Radiological Protection (ICRP). Basic anatomical and physiological data for use in radiological protection: reference values. In: Valentin J (ed.). Annals of the ICRP. ICRP Publication, Oxford, 89.

Vales G, Rubio L and Marcos R, 2015. Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells. Nanotoxicology, 9, 568–578.

Vasantharaja D, Ramalingam V and Reddy GA, 2015. Oral toxic exposure of titanium dioxide nanoparticles on serum biochemical changes in adult male Wistar rats. Nanomedicine Journal, 2, 46–53.

Verleysen E, Waegeneers N, Brassinne F, De Vos S, Jimenez IO, Mathioudaki S and Mast J, 2020. Physicochemical characterization of the pristine E171 food additive by standardized and validated methods. Nanomaterials (Basel), 10, 592.

Verleysen E, Waegeneers N, De Vos S, Brassinne F, Ledecq M, Van Steen F, Andjelkovic M, Janssens R, Mathioudaki S, Delfosse L, Machiels R, Cheyns K and Mast J, 2021. Physicochemical characterization of nanoparticles in food additives in the context of risk identification. EFSA supporting publication 2021:EN-9992. 31 pp. https://doi.org/10.2903/sp.efsa.2021.EN-9992

Viennois E and Chassaing B, 2018. First victim, later aggressor: how the intestinal microbiota drives the proinflammatory effects of dietary emulsifiers? Gut Microbes, 1–4.
Vila L, Garcia-Rodriguez A, Marcos R and Hernandez A, 2018. Titanium dioxide nanoparticles translocate through differentiated Caco-2 cell monolayers, without disrupting the barrier functionality or inducing genotoxic damage. Journal of Applied Toxicology: JAT, 38, 1195–1205.
Wallin H, Kyjovska ZO, Poulsen SS, Jacobsen NR, Saber AT, Bengtson S, Jackson P and Vogel U, 2017. Surface modification does not influence the genotoxic and inflammatory effects of TiO_2 nanoparticles after pulmonary exposure by instillation in mice. Mutagenesis, 32, 47–57.
Wang JJ, Sanderson BJS and Wang H, 2007. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutation Research, 628, 99–106.
Wang S, Hunter LA, Arslan Z, Wilkerson MG and Wickliffe JK, 2011. Chronic exposure to nanosized, anatase titanium dioxide is not cyto- or genotoxic to Chinese hamster ovary cells. Environmental and Molecular Mutagenesis, 52, 614–622.
Wang Y, Cui H, Zhou J, Li F, Wang J, Chen M and Liu Q, 2015. Cytotoxicity, DNA damage, and apoptosis induced by titanium dioxide nanoparticles in human non-small cell lung cancer A549 cells. Environmental Science and Pollution Research International, 22, 5519–5530.
Wang X, Liu Y, Wang J, Nie Y, Chen S, Hei TK, Deng Z, Wu L, Zhao G and Xu A, 2017. Amplification of arsenic genotoxicity by TiO_2 nanoparticles in mammalian cells: new insights from physicochemical interactions and mitochondria.
Wang H, Ni J, Guo X, Zhou T, Ma X, Xue J and Wang X, 2018. Shelterin differentially respond to oxidative stress induced by TiO_2-NPs and regulate telomere length in human hepatocytes and hepatocarcinoma cells in vitro. Biochemical and Biophysical Research Communications, 503, 697–702.
Wang J, Wang J, Liu Y, Nie Y, Si B, Wang T, Waqas A, Zhao G, Wang M and Xu A, 2019. Aging-independent and size-dependent genotoxic response induced by titanium dioxide nanoparticles in mammalian cells. Journal of Environmental Sciences (China), 85, 94–106.
Warheit DB, Hoke RA, Finlay C, Donner EM, Reed KL and Sayes CM, 2007. Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. Toxicology Letters, 171, 99–110.
Warheit DB, Brown SC and Donner EM, 2015a. Acute and subchronic oral toxicity studies in rats with nanoscale and pigment grade titanium dioxide particles. Food and Chemical Toxicology, 84, 208–224.
Warheit DB, Boatman R and Brown SC, 2015b. Developmental toxicity studies with 6 forms of titanium dioxide test materials (3 pigment-different grade & 3 nanoscale) demonstrate an absence of effects in orally-exposed rats. Regulatory Toxicology and Pharmacology, 73, 887–896.
Weir A, Westerhoff P, Fabricius L, Hristovski K and von Goetz N, 2012. Titanium dioxide nanoparticles in food and personal care products. Environmental Science and Technology, 46, 2242–2250.
Woodruff RS, Li Y, Yan J, Bishop M, Jones MY, Watanabe F, Biris AS, Rice P, Zhou T and Chen T, 2012. Genotoxicity evaluation of titanium dioxide nanoparticles using the Ames test and Comet assay. Journal of Applied Toxicology, 32, 934–943.
Xu A, Chai Y, Nohmi T and Hei TK, 2009. Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. Particle and Fibre Technology, 6, 3.
Xu J, Shi H, Ruth M, Yu H, Lazar L, Zou B, Yang C, Wu A and Zhao J, 2013. Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. PLoS ONE, 8, e70618. https://doi.org/10.1371/journal.pone.0070618. Collection 2013.
Yan J, Wang D, Li K, Chen Q, Lai W, Tian L, Lin B, Tan Y, Liu X and Xi Z, 2020. Toxic effects of the food additives titanium dioxide and silica on the murine intestinal tract: mechanisms related to intestinal barrier dysfunction involved by gut microbiota. Environmental Toxicology and Pharmacology, 80.
Yang J, Luo M, Tan Z, Dai M, Xie M, Lin J, Hua H, Ma Q, Zhao J and Liu A, 2017. Oral administration of nano-titanium dioxide particle disrupts hepatic metabolic functions in a mouse model. Environmental Toxicology and Pharmacology, 49, 112–118.
Yang S, Xiong F, Chen K, Chang Y, Bai X, Yin W, Gu W, Wang Q, Li J and Chen G, 2018. Impact of titanium dioxide and fullerenol nanoparticles on caco-2 gut epithelial cells. Journal of Nanoscience and Nanotechnology, 18, 2387–2393.
Yang B, Wei J, Ju P and Chen J, 2019. Effects of regulating intestinal microbiota on anxiety symptoms: a systematic review. Gen Psychiatr, e100056, 17.
Yin C, Zhao W, Liu R, Liu R, Wang Z, Zhu L, Chen W and Liu S, 2017. TiO_2 particles in seafood and surimi products: attention should be paid to their exposure and uptake through foods. Chemosphere, 188, 541–547.
Yu X, Hong F and Zhang YQ, 2016. Cardiac inflammation involving in PKCε or ERK1/2-activated NF-κB signalling pathway in mice following exposure to titanium dioxide nanoparticles. Journal of Hazardous Materials, 313, 68–77.
Zhang S, Jiang X, Cheng S, Fan J, Qin X, Wang T, Zhang Y, Zhang J, Qiu Y, Qiu J, Zou Z and Chen C, 2020. Titanium dioxide nanoparticles via oral exposure leads to adverse disturbance of gut microecology and locomotor activity in adult mice. Archives of Toxicology, 94, 1173–1190.
Zhao Y, Tang Y, Chen L, Lv S, Liu S, Nie P, Aguilar ZP and Xu H, 2020. Restraining the TiO_2 nanoparticles-induced intestinal inflammation mediated by gut microbiota in juvenile rats via ingestion of *Lactobacillus rhamnosus* GG. Ecotoxicology and Environmental Safety, 206.

Zhou Y, Hong F, Tian Y, Zhao X, Hong J, Ze Y and Wang L, 2017. Nanoparticulate titanium dioxide-inhibited dendritic development is involved in apoptosis and autophagy of hippocampal neurons in offspring mice. Toxicology Research, 6, 889–901.

Zijno A, De Angelis I, De Berardis B, Andreoli C, Russo MT, Pietraforte D, Scorza G, Degan P, Ponti J, Rossi F and Barone F, 2015. Different mechanisms are involved in oxidative DNA damage and genotoxicity induction by ZnO and TiO_2 nanoparticles in human colon carcinoma cells. Toxicology in Vitro: An International Journal Published In Association With BIBRA, 29, 1503–1512.

Zijno A, Cavallo D, Di Felice G, Ponti J, Barletta B, Butteroni C, Corinti S, De Berardis B, Palamides J, Ursini CL, Fresegna AM, Ciervo A, Maiello R and Barone F, 2020. Use of a common European approach for nanomaterials' testing to support regulation: a case study on titanium and silicon dioxide representative nanomaterials. Journal of Applied Toxicology.

Zirak RG, Lotfi A and Moghadam MS, 2016. Effects of the interaction of nanoanatase TiO_2 with bleomycin sulfate on chromosomal abnormalities in vivo. International Journal of Advanced Biotechnology and Research, 7, 1094–1108.

Abbreviations

8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
ABC	aberrant crypt
ACF	aberrant crypt foci
AChE	acetylcholinesterase
ADI	accepted daily intake
AFM	atomic force microscopy
AGD	anogenital distance
ALB	albumin
ALT	alanine aminotransferase
ALP	alkaline phosphatase
ANOVA	analysis of variance
ANSES	French Agency for Food, Environment and Occupational Health and Safety
AOM	azoxymethane
AST	aspartate aminotransferase
AUC	area under the curve
BAL	bronchoalveolar lavage
BChE	butyrylcholinesterase
BSFGE	biased sinusoidal field gel electrophoresis
BTB	blood–testis barrier
BUN	blood urea nitrogen
bw	body weight
CA	chromosomal aberration
CAC	colitis-associated colorectal cancer
CAT	catalase
ccWG	cross-cutting Working Group
CD	circular dichroism
CK	creatine kinase
COX	cycloxygenase
CREA	creatinine
CY	cyclophosphamide
DBP	diastolic blood pressure
DG	dentate gyrus
DMH	dimethylhyrazine
DOX	doxorubicin
DSS	dextran sulfate sodium
ECHA	European Chemicals Agency
EFSA	ANS EFSA Panel on Food Additives and Nutrient sources added to Food
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil-activating protein-78
EOGRT	extended one-generation reproduction toxicity
EXAFS	extended X-ray absorption fine structure
FACS	flow cytometry analysis

FAF	Food Additives and Flavourings
FCS	food categorisation system
FFA	free fatty acid
Fpg	formamidopyrimidine DNA glycosylase
GALT	gut-associated lymphoid tissue
GC–MS/MS	gas chromatography–tandem mass spectrometry
GD	gestation day
GEF	global evaluation factor
GI	gastrointestinal
GIT	gastrointestinal tract
GLB	globulin
GLP	Good laboratory practice
GM-CSF	granulocyte-macrophage colony-stimulating factor
GNPD	Global New Products Database
GPx	glutathione peroxidase
GSH	glutathione
GSP	glycated serum protein
H&E	haematoxylin and eosin
HBDH	α-hydroxybutyrate dehydrogenase
HBGV	health-based guidance value
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HMC	hydroxypropyl methylcellulose
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HR	heart rate
IBO	interested business operator
ICP-AES	inductively coupled plasma atomic emission spectroscopy
ICP-DRC-MS	inductively coupled plasma dynamic reaction cell mass spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectroscopy
Ig	immunoglobulin
IL-6	interleukin-6
i.p.	intraperitoneal
i.v.	intravenous
JAK2	Janus protein tyrosine kinase 2
KLH	keyhole limpet haemocyanin
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LOD	limit of detection
LOQ	limit of quantification
LPO	lipid peroxidation
m-SRXRF	micro-synchrotron radiation X-ray fluorescence
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MDA	malondialdehyde
MMP	mitochondrial membrane potential
MN	micronuclei
MoS	margin of safety
MP	microparticulate
MPL	maximum permitted level
MS/MS	tandem mass spectrometry
MWM	Morris water maze
NBT	nitro blue tetrazolium
NGAL	neutrophil gelatinase-associated lipocalin
NOAEL	no-observed-adverse-effect level
NSC	nanoscale considerations

NVWA	Netherlands Food and Consumer Product Safety Authority
OECD	glycated serum protein
OFT	open field test
OGTT	oral glucose tolerance test
PA	passive avoidance
PBMC	peripheral blood mononuclear cell
PC	protein carbonylation
PCE	polychromatic erythrocyte
PGC-1α	PPAR-gamma coactivator 1 alpha protein
PND	postnatal day
PPAR-γ	peroxisome proliferator-activated receptor-gamma
PTX	paclitaxel
qRT-PCR	quantitative real-time polymerase chain reaction
QS	*quantum satis*
RAC	Committee for Risk Assessment
RP	reference point
SBP	systolic blood pressure
SCFA	short-chain fatty acid
SEM	*scanning electron microscopy*
SOD	superoxide dismutase
spICP-MS	single particle inductively coupled plasma mass spectrometry
SSB	single-strand break
STAT6	signal transducers and activators of transcription 6
STEM-EDX	Scanning transmission electron microscopy coupled to energy dispersive X-ray
SOCS1	suppressors of cytokine signalling
TAS	total antioxidant status
TBAR	total antioxidant capacity
TBARS	thiobarbituric acid reactive substances
TBILI	total bilirubin
TC	total cholesterol
T/D	transformation/dissolution
TEM	transmission electron microscopy
TEM-EDX	transmission electron microscopy energy-dispersive X-ray spectroscopy (
TG	triglyceride
TNF-α	tumour necrosis factor-alpha
TP	total protein
T-SOD	total superoxide dismutase
UA	uric acid
WBC	white blood cells
XRD	X-ray diffraction

Appendix A – Search methodology for the literature search

Appendix B – Criteria for inclusion and exclusion applied to screening of publication retrieved in the literature search

Appendix C – Approach for assessing toxicokinetic and toxicity studies

Appendix D – Approach for assessing genotoxicity studies

Appendix E – Advice from EFSA ccWG Nanotechnology: Nanoscale considerations for the assessment of the study design and study results of TiO_2 toxicity studies

Appendix F – List of *in vivo* toxicokinetic and toxicity studies retrieved from the literature search

Appendix A–F can be found in the online version of this output (in the 'Supporting information' section): https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2021.6585

Appendix G – Description of test materials, scoring for nanoscale consideration and internal exposure for toxicokinetic studies

Reference	Test material	Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E	Internal exposure
Talamini et al. (2019)	E 171 (35% nano), anatase, 201 nm in suspension (NTA)	1	Quantitative analysis in tissues with methodology reliable with some limitations
Coméra et al. (2020)	E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles < 100 nm	1	Quantitative analysis in tissue; methodology reliable with some limitations
Riedle et al. (2020)	E 171, anatase, 119 nm (TEM)	1	Qualitative analysis in tissues, methodology reliable with some limitations
Pele et al. (2015)	E 171, anatase, d50 = 260 nm	1	Qualitative analysis in blood, methodology highly reliable
Guillard et al. (2020)	E 171, anatase, 20–440 nm (mean 104.9 nm (SEM-EDX), 55% nanoparticles	1	Quantitative analysis in tissues, methodology reliable with some limitations
Disdier et al. (2015)	TiO_2 NPs, P25 (15–24 nm)	1	Quantitative analysis in blood and tissues, methodology reliable with some limitations
Kreyling et al. (2017a)	TiO_2 NPs, anatase, 50 nm (TEM), purity unknown	1	Quantitative analysis in blood and tissues; methodology highly reliable
Geraets et al. (2014)	NM-100 (50–150 nm) and NM-102 (21–22 nm)	1	Quantitative analysis in tissues; methodology highly reliable
Tassinari et al. (2014)	TiO_2 NPs, anatase, two different morphologies: spherical shape with a size 20–60 nm (TEM) and irregular 60–40 nm (TEM)	1	Quantitative analysis in tissues; methodology highly reliable
Hendrickson et al. (2016)	TiO_2 NPs, anatase, 20–25 nm (TEM)	1	Quantitative analysis in blood and tissue, methodology reliable with some limitations
Ammendolia et al. (2017)	TiO_2 NPs, anatase, spherical shape 20–60 nm and irregular shape 40x60 nm (TEM)	1	Quantitative analysis in tissues, methodology highly reliable
Kreyling et al. (2017b)	TiO_2 NPs, anatase, 50 nm (TEM), purity unknown	1	Quantitative analysis in blood and tissue, methodology highly reliable
Hendrickson et al. (2020)	TiO_2 NPs, rutile, needle- or rod-like shape, 5 × 30 nm (TEM)	1	Particles identified in tissues by EM, methodology reliable with some limitations
Chen et al. (2020a,b)	TiO_2 NPs, anatase, 29 nm (SEM)	1	Quantitative analysis in blood and tissue, methodology reliable with some limitations
Heringa et al. (2018)	TiO_2 particles	Not experimental study	Quantitative analysis in tissue, methodology highly reliable
Peters et al. (2020)	TiO_2 particles	Not experimental study	Quantitative analysis in tissue, methodology highly reliable

NTA: nanoparticle tracking analysis; SEM: scanning electron microscopy: TEM: transmission electron microscopy.

Appendix H – Description of toxicity studies classified with reliability 1 and 2

General and organ toxicity studies

E 171

Mice

Talamini et al. (2019)

Test material: E 171 (35% nano), anatase, 201 nm in suspension (NTA).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure examined: quantitative analysis in tissues with methodology reliable with some limitations.

Eight-week-old male NFR mice were randomly divided into two groups (22 animals/group). One group was orally treated with 5 mg E 171/kg bw, freshly dispersed in water. The other group was treated with water (vehicle control). E 171 or water was slowly dripped with a pipette into the mice mouths, allowing each drop to be swallowed. Mice were treated 3 days/week for 3 weeks for a total of nine treatments in 21 days. According to the authors, this treatment schedule resulted in the mice receiving an average daily dose of 2 mg E 171/kg body weight (bw).

On day 21, three days after the last dose, mice were anaesthetised with 5% isoflurane and blood was collected in heparinised tubes from the retro-orbital plexus. They were then killed by cervical dislocation and liver, spleen and whole intestine excised for either histological examination (4/group); superoxide determinations after first snap freezing in dry ice and storing at –80°C (4/group) or gene expression analyses (10/group).

A statistically significant increase in 'necroinflammatory area' was observed in treated mouse liver based on bright-field haematoxylin and eosin (H&E) images acquired with an Aperio Scanscope System CS2 microscope and an ImageScope program (the areas occupied by necroinflammatory foci in the liver were manually identified by three different operators blinded to treatment allocation).

Statistically significant increases in apparent superoxide levels were reported in stomach (~ 50%) and whole intestine (~ 25%) – but not liver – according to the authors, through nitro blue tetrazolium (NBT) reduction.

Statistically significant increases in IL-1β – but not TNF-α or IL-10 – mRNA transcripts were reported in both stomach (~ 75%) and whole intestine tissues (~ 75%), but not liver tissues. A statistically significant reduction in liver IL-10 mRNA transcript expression was observed (~ 40%). These data are based on quantitative real-time polymerase chain reaction (qRT-PCR) methodology.

The Panel noted that this study was limited to one dose group and that the increase in necroinflammatory area was not accompanied by additional endpoints indicative of evidence for liver injury. Furthermore, the Panel noted that the histological examination of the liver was performed only on two male mice in the control group and four male mice in the group exposed to E 171. Considering that this type of inflammatory foci (inflammatory cell infiltrates with adjacent necrotic hepatocytes with distinct eosinophilic cytoplasm) of variable size are known to be present in not-treated mice, a conclusion of the relation to the treatment of these lesions cannot be drawn. The biochemical changes in the stomach and small intestine were considered indicative for increases in oxidative stress and inflammation and adaptive, but not evidence of adversity.

Rats

Talbot et al. (2018)

Two test materials: E 171, anatase, 118 nm (TEM), 45% nanoparticles; 2) TiO_2NPs (NM-105), anatase/rutile, 15–24 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure not examined.

Adult male Wistar rats (175–200 g) were randomly divided into three groups (n = 8) and daily administered by gavage E 171 (10 mg/kg bw per day), NM-105 (10 mg/kg bw per day) or vehicle

(water) for 7 days. In the second series of experiments, rats were randomly divided into three groups (n = 10) and exposed for 60 days to E 171 at doses of 10 mg/kg bw per day or 0.1 mg/kg bw per day, through the drinking water, or water alone for the control animals. The stock suspensions of E 171 or TiO_2 NPs in water were prepared fresh prior to each experiment. At the end of study rats were sacrificed and the caecal contents collected for analysis of short-chain fatty acids (SCFAs) and tissues from the small intestine and distal colon sampled for mucin O-glycosylation. SCFAs were assessed by gas–liquid chromatography. Jejunal and/or ileal and distal colonic mucosa were scraped and the mucins solubilised and purified, permethylated oligosaccharides were analysed by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry.

The Panel considered that the results indicate that TiO_2 did not modify caecal short-chain fatty acid profiles (involved in the regulation of intestinal mucin MUC2 expression) and gut mucin O-glycosylation patterns (it influences the cohesive properties of mucus and hence its protective function), indicating the absence of a mucus barrier impairment. The absence of mucin O-glycan alterations indicates that the protective function of mucus against particle uptake remained intact, even after subchronic oral exposure to E 171 (NM-105 was not tested at 60 days).

Han et al. (2020a)

Test material: E 171, anatase, 150 nm (dynamic light scattering (DLS)),

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure: quantitative analysis in tissues; methodology not reliable.

Sprague–Dawley rats (10/sex per group) received by gavage 0, 10, 100 or 1,000 mg E 171/kg bw per day for 90 day. The study was conducted according to OECD TG 408. No mortality was observed. Clinical appearance and body weights of the treated males and females were comparable with those in the controls. Feed intake in high-dose males was statistically significantly increased from day 11 onwards with exception for days 39 and 74, the difference to controls ranging from 6% to 10%. The statistically significant increase in feed intake was also seen in the low-dose males on days 11, 18 and 88 (+8%, +5% and +7%, respectively). Haematological examination revealed a slight but statistically significant decrease in relative lymphocyte count in low- and high-dose males (8% at each dose). No differences relative to controls were reported in all treated groups of both sexes in clinical chemistry, absolute and relative organ weights or type and incidence of macroscopic and histopathological findings. In the stomach from high-dose rats (sex not informed), the test material was observed on the surface and in mucosa. The authors referred to this finding as 'E 171 accumulation in the stomach wall'. Analysis of Ti amount in the colon revealed a higher amount of the element in the high-dose group relative to control, while Ti amount in the kidney and spleen were similar to those in the controls.

The Panel noted that oral administration of E 171 in doses amounting to 1,000 mg/kg bw per day to rats had no adverse effects on general toxicity endpoints.

TiO_2 NPs or TiO_2 containing a fraction of nanoparticles

Rats

Warheit et al. (2015a)

Test material: TiO_2 (11% nano), rutile, d50 = 173 nm (TEM), purity unknown. Two samples were tested, one was a research grade and the other a commercial grade of the pigment; both having the same physico-chemical characterisation.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

In a 28-day repeated dose study (OECD TG 407), two groups of young adult male rats (n = 5/group) were gavaged with TiO_2 (11% nano) as a suspension (4 mL/100 g bw) at 24,000 mg/kg bw per day. A control group (n = 5) was administered nanopure water (vehicle). Clinical pathology endpoints were evaluated after the end of the 29-day treatment.

No clinical signs of toxicity, and no effects on body weight or nutritional and clinical pathology parameters were observed. No adverse effects on organ weights (adrenal glands, brain, epididymides,

heart, kidneys, liver, spleen, testes and thymus) and no gross or microscopic changes were reported. The authors reported (data not shown) that the evidence of the test substance was observed in intestinal lymphoid tissue of the treated group through microscopic examination but this was not considered to be an adverse effect. They concluded that the no-observed-adverse-effect level (NOAEL) was 24,000 mg/kg bw per day, the only dose tested.

The Panel noted that a 90-day toxicity study (OECD TG 408) with TiO_2 (21% nano) rutile coated with Al_2O_3 is also described in this publication. Since this tested material was TiO_2 coated with Al_2O_3 and was not representative of E 171, the data pertaining to this material was not considered relevant for the assessment of E 171 (according to Appendix B).

Vasantharaja et al. (2015)

Test material: TiO_2 NPs, anatase/rutile, < 100 nm, purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Adult male Wistar rats divided in groups (n = 6) received TiO_2 NPs suspended in 0.9% saline by gavage at doses of 0, 50 and 100 mg/kg bw per day for 14 consecutive days.

Serum was obtained at the end of the experiment and different clinical biochemistry parameters were measured. Total protein (TP), albumin (ALB), globulin (GLB), cholesterol, triglycerides (TGs) and high-density lipoprotein (HDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBILI), blood urea nitrogen (BUN), uric acid (UA) and creatinine (CREA) as well as glucose

The following changes were observed: glucose increased in the treated groups (1.17- and 1.19-fold, at 50 and 100 mg/kg bw, respectively; cholesterol increased (1.25- and 1.5-fold, respectively), with mild elevation of HDL (1.13-fold) at the high dose; TG decreased to 64% of the control in both groups no significant difference in ALB, GLB and TBILI. TP changes while statistically significant, were both very limited (< 10% change) and not dose dependent (with a decrease to 92% of the control at the low dose and an increase to 1.06% at the high dose).

Serum AST levels were significantly increased only at the high treatment dose by ca 10%, while ALT levels were unchanged. A decrease (by ca. 10%) of ALP activity was observed in both the treated groups. BUN increased at both doses (1.17- and 1.21-fold at low and high doses, respectively) and UA increased by 1.3- and 1.5-fold, at low and high doses, respectively, but the statistical significance of these changes was either absent or weak ($p < 0.05$). There was no change of CREA.

The Panel noted a single major change, reduction of serum TG in both the treated groups. The Panel considered that no adverse effects of toxicological significance were observed.

El-Din et al. (2019)

Test material: TiO_2 NPs, anatase/rutile, < 100 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

In a study investigating whether curcumin could ameliorate cardiotoxic (and genotoxic) effect of TiO_2 NPs male albino rats (strain not informed; n = 8/group) received by gavage either saline (vehicle control A), corn oil (vehicle control B), 200 mg curcumin/kg bw in corn oil, 1,200 mg TiO_2 NPs/kg bw per day in saline or 200 mg curcumin/kg bw per day in corn oil and 1,200 mg TiO_2 NPs/kg bw per day in saline for 90 days (El-Din et al., 2019). Additionally, an untreated control group (n = 8) was included. In this assessment only the results from the TiO_2 NPs and the control groups are presented. Histopathological examinations were made on heart ventricular tissue, accompanied by some immunohistochemical staining. Photomicrographs of the H&E-stained samples revealed wide intercellular spaces between cardiomyocytes, vacuolation of cytoplasm, deeply stained homogenous acidophilic cytoplasm with fading striation or pale acidophilic material indicative of oedema in some cardiomyocytes, severely congested capillaries, extravasation, pyknotic and peripherally displaced nuclei of cardiomyocytes, prominent mononuclear cell infiltration of the tissue in the TiO_2 NPs group. Immunohistochemically stained specimens of the heart ventricle revealed a statistically significant increased per cent areas for collagen and anti-3-nitrotyrosine staining parts and a statistically

significantly decreased per cent area for anti-desmin in the TiO_2 NPs group as compared to the untreated or vehicle control groups. The authors concluded that TiO_2 NPs could cause cardiac toxicity. The Panel noted that the number of stained samples examined per group were not reported and information of blinding of samples was not given. Based on the limited reporting of the morphological changes in the heart ventricular tissue, the Panel was not able to conclude on the relationship between the reported histopathological changes and treatment with TiO_2 NPs.

TiO_2 NPs < 30 nm

Mice

Hu et al. (2015)

Test material: TiO_2 NPs, anatase, 25.6 nm (TEM, SEM), purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure examined: quantitative analysis in blood and tissues; methodology with important flaws

In a study designed to explore potential endocrine effects of TiO_2 NPs, 6-week-old CD-1 male mice (25.37 $\pm$ 0.71 g; n = 10/group) were acclimatised for 7 days and provided with autoclaved water and rodent diet ad libitum then randomly divided into three groups: control (PBS vehicle), 64 mg TiO_2 NPs/kg bw per day and 320 mg TiO_2 NPs/kg bw per day and dosed daily for 14 weeks by gavage.

Body weights of mice and food intakes were determined every week and biweekly, respectively.

Every 2 weeks, mice were fasted for 16 h prior to daily administration of TiO_2 NPs and blood was collected from the tail vein to measure the plasma glucose level. If plasma glucose was increased in comparison to control group, mice plasma glucose was tested for 4 weeks. After 14 weeks, blood was also collected from the heart.

Ti concentration was measured in organs (liver lobes, spleen, middle part of small intestine, gastrocnemius muscle, middle part of the kidney and pancreas) using ICP-OES, and Ti particles in the tissue were detected by EDXA. The Panel noted that the analytical technique (ICP-OES) was not appropriate for a reliable Ti quantification in tissues.

Ti concentrations were in both the treated groups increased in all organs, with no significant difference between the two dose groups. Ti concentration in kidney and small intestine was the highest. No significant difference in body weights or food intake was observed between the 3 groups. However, plasma glucose levels (tail vain) were increased from week 10 in both TiO_2 NPs groups. The plasma glucose concentration in heart blood at termination was also statistically significantly increased. There were no changes in the concentrations of plasma insulin, blood TG, free fatty acid (FFA), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) or total cholesterol (TC) in any group. Levels of total superoxide dismutase (T-SOD) and glutathione (GSH) were significantly reduced and the level of MDA was significant increased for both dose group in serum and liver. The authors concluded that oral exposure to TiO_2 NPs increased reactive oxygen species. No apoptotic pancreatic β-cells were observed.

In an oral glucose tolerance test (OGTT), a significant increase in plasma glucose levels was observed in the treated animals compared to control group, but no differences in plasma insulin levels at 30, 60 and 120 min were observed.

At week 14, western blot results showed that the phosphorylation of IRS1 (Ser307) was increased and phosphorylation of Akt (Ser473) was reduced in the liver in both the dose groups. The serum levels of tumour necrosis factor-alpha (TNF-α) were significantly increased for both groups compared to control. IL-6 was significantly increased in the serum of the high-dose group. Phosphorylation of JNK1 and p38 MAPK was significantly increased in the liver in both dose groups.

According to the authors, this study indicates that oral administration of TiO_2 NPs increases plasma glucose in mice, in fasting state and after OGTT without showing a dose response.

According to the authors, TiO_2 NPs increased ROS by activating an inflammatory response and MAPK pathways, which induce the insulin resistance and cause an increased plasma glucose levels in mice.

The Panel considered that the oral administration of TiO_2 NPs (25.6 nm) at both doses, 64 and 320 mg/kg bw per day leads to increases in fasting state plasma glucose, and also to increases in glucose

levels in a glucose tolerance test without showing a dose response and without differences in plasma insulin levels, indicating inconsistency between the measured outcomes.

Yu et al. (2016)

Test material: TiO_2 NPs, anatase, 5–6 nm (further information on characterisation from Hu et al., 2011).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

In a study assessing effects of TiO_2 NPs on the heart, female CD 1 (ICR) mice (initial body weight 20 ± 2 g, n = 20/group) received by gavage 0, 2.5, 5 or 10 mg TiO_2 NPs/kg bw per day for 90 days. The endpoints evaluated were body weight gain, absolute and relative heart weight (for 20 mice per group), morphology of the heart, activities of ATPases and protein expression of CAMK II, NCX-1, α-1AR and β-1AR in the heart tissue by ELISA (each parameter for 5 mice per group). The results for endpoints related to the immunological system are not included. Body weight gain was statistically significantly decreased in a dose-dependent manner up to 30.3% in all TiO_2 NPs groups. Mean absolute heart weights were comparable with those of the control group but the relative heart weights were increased with increasing dose and the difference to the control group reached statistical significance at the high dose. Microscopic examination of H&E-stained heart tissue revealed fragmentation, interstitial spaces or disordered myocardial fibre arrangement, fatty degeneration or necrosis at all doses, and cardiomyocyte hypertrophy, myocardial haemorrhage or hyperaemia and focal inflammatory cell infiltration in myocardial interstitium at mid- and high-dose groups. There were statistically significant decreases in the activities of cardiac Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPse at all doses and of Ca^{2+}-ATPase in mid and high doses. The decreases were dose related. The decreases at the high dose were approximately 25%, 26% and 23% for Ca^{2+}-ATPase, Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase, respectively, as estimated by the Panel. The Panel noted that reporting of morphological changes in the heart was based solely on the presented photomicrographs from the heart sections but the incidence and severity scores of the morphological changes were not presented. Based on this limited reporting, the Panel was not able to conclude on the decreased body weight gain and the relationship between the reported histological changes and treatment with TiO_2 NPs (5–6 nm). The Panel further noted that the decreases in activities of ATPases were not indicative for an impaired cardiac pump function (Akera and Brody, 1978; Dostanic et al., 2004; Griffiths et al., 2009; Figtree et al., 2012).

Hong et al. (2016)

Test material: TiO_2 NPs, anatase, 5–6 nm (further information on characterisation from Hong et al., 2014).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

CD-1 (ICR) male mice were divided into four groups (n = 40) and administered TiO_2 NPs by gavage at doses of 0, 2.5, 5 and 10 mg/kg bw per day for 90 days.

A dose-dependent decrease in body weight gain was observed at all tested doses (approx. 5%, 5% and 7% decrease in body weight gain compared to control at 2.5, 5 and 10 mg/kg bw per day, respectively), with statistically significant differences at the two highest doses. Relative liver weights increased by ~ 10–15% compared to control however, absolute liver weights were unchanged. According to the authors, histopathology 'showed dose-related severity of liver injury, such as hyperaemia, lymphocyte infiltration and necrosis'. However, the Panel noted that quantitative data (incidence and severity) were not reported and there may be operator bias in selection of images.

According to the authors, the inflammatory response in the liver was demonstrated by dose-dependent increases in protein expression of cycloxygenase (COX, ca. 30% and 100% at 5 and 10 mg/kg, respectively), neutrophil gelatinase-associated lipocalin (NGAL, by ca. 50 and 80%) and epithelial neutrophil-activating protein-78 (ENA-78, by ca. 23%), and decreased protein expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ, to ca. 29 and 27% of control). The

decreased expression of PPAR-gamma coactivator 1 alpha protein (PGC-1α, to ca. 82 and 61% of control) indicates, according to the authors, possible decreased capacity in mitochondrial metabolism.

The ET-1 and P2X7 levels were not significantly different among the four groups. Western blot analysis of biomarkers of inflammation showed increased expression of IL-6, Janus protein tyrosine kinase 2 (JAK2), signal transducers and activators of transcription 6 (STAT6), COX, NGAL and ENA-78, and decreased expression of suppressors of cytokine signalling (SOCS1), PPAR-γ and PGC-1α. According to the authors, 'the JAK–STAT pathway may play an important role in nano-TiO_2-induced liver injury'.

The Panel noted that the histopathological data in the liver was not accompanied by any other confirmatory investigations (e.g. clinical chemistry) and considered the effects reported in this study as likely an hepatic inflammatory response to TiO_2 NPs (5–6 nm).

Yang et al. (2017)

Test material: TiO_2 NPs, mixture (anatase and rutile), 21 nm (the Panel noted that from the product number catalogue indicated in the publication, it is P25).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure examined: particles identified in liver; methodology with important flaws.

Male 5- to 7-week-old C57/Bl6 mice (22–25 g, 5/group) were treated by gavage once daily for 14 days with either vehicle control (0.5% CMC-Na, not defined), 250 mg TiO_2 NPs/kg bw or 500 mg TiO_2 NPs/kg bw. The Panel noted there was no randomisation of animals in this study.

After 14 days, blood was collected from the retro-orbital vessel. Mice were then killed by CO_2 asphyxiation. Two samples of each liver from three mice in each group were examined via TEM, with the remainder either fixed for histopathological examination or frozen for later analyses of gene expression.

There were no significant effects of TiO_2 NPs on relative liver weight, serum ALT, AST, ALP and total bile acid levels. Conjugated bilirubin (direct) and bilirubin (indirect) serum levels were statistically significantly elevated ~ 2.5- to 3-fold in the highest dose group. However, no histopathological changes were observed in the liver (livers from three randomly selected mice from each experimental group were examined).

The authors reported increases in particles, the number of mitochondria and oedema of the endoplasmic reticulum in hepatocytes based on analysis by TEM in both the dose groups. However, none of these data were analysed quantitatively.

qRT-PCR and western blotting on liver tissues identified significant increases in the expression of selected genes (Oatp1, Mrp3, Cyp2b10 and Cyp2c37) with increasing dose of TiO_2 NPs (21 nm).

The Panel noted the increases in serum bilirubin levels along with structural changes in hepatocytes which were not quantified. However, these increases occurred in the absence of any changes in relative liver weight, changes in other serum markers for liver injury or quantitative histopathological changes in the liver. Changes in the hepatic expression of selected genes were considered as either spontaneous or adaptive, but not evidence of adversity.

Rats

Chen et al. (2015a)

Test material: TiO_2 NPs, anatase 24 nm (TEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Four-week-old healthy Sprague–Dawley rats (n = 10 per group) were randomised into experimental and control groups, with five male and five female rats in each group. Suspensions in ultrapure water of TiO_2 NPs (0, 2, 10 and 50 mg/kg bw per day), glucose (1.8 g/kg bw per day) and TiO_2 NPs (0, 2, 10, 50 mg/mg bw per day) + glucose (1.8 g/kg bw per day) were gavaged daily for 30 or 90 consecutive days. The animals were kept under standard environmental conditions. In this assessment only the results from the TiO_2 NPs and the control groups are presented.

During the experiments, no significant changes in the body weight and food intake of the exposed rats were found and no mortality was observed.

After 30 days or 90 days, animals were weighed and sacrificed. Blood samples were collected for haematological and clinical chemistry investigations. The liver, kidney, spleen, testicle, ovary and heart were collected.

The relative kidney weight was increased in male rats only in the 2 and 10 mg/kg bw per day groups, not in the 50 mg/kg bw per day group; the increase being statistically significant, but biologically not relevant (about 10%). No difference in heart body weight was recorded after 30 or 90 days.

No effect was seen on the relative heart weight. No adverse effect on heart rate and blood pressure was noted. Some laboratory parameters were changed (α-hydroxybutyrate dehydrogenase (HBDH) decreased at 90 days in males in the dose group of 50 mg/kg bw per day, CK decreased at 90 days in females in the dose group of 10 mg/kg bw per day, serum BUN increased at 90 days in males the dose group of 10 mg/kg bw per day without increased serum creatinine, serum creatinine decreased at 90 days in males the dose group of 50 mg/kg bw per day), however, the Panel considered of no toxicological relevance.

Relative organ weight of liver and spleen were unchanged in all groups.

No histopathological findings were reported, except for the liver for which the authors reported 'edema, fatty degeneration and necrosis were evident in all the 50 mg/kg bw per day treated groups'. However, the images shown are too small to independently assess the reported findings. In addition, serum enzyme activities reflecting liver injury (the aminotransferases ALT, AST and ALT/AST) were unchanged in females or males exposed to TiO_2 NPs, irrespective of the dose, and this does not confirm the histopathological changes reported by the authors. Mostly random, modest changes in total bilirubin values were observed across the control and treated groups of both sexes. The authors considered that decreased total bilirubin values in the female groups treated with 10 and 50 mg TiO_2 NPs/kg bw per day was a sign of liver dysfunction, but the Panel did not agree since there is no liver injury which is known to result in hypobilirubinaemia.

The Panel noted that no TiO_2 measurements were performed in blood or tissues. The Panel considered that no adverse effects were identified in this study up to a dose of 50 mg TiO_2 NPs (24 nm)/kg bw per day.

Chen et al. (2015b)

Test material: TiO_2 NPs, anatase, 24 nm (TEM) (physico-chemical properties were described in detail at Chen et al., 2015a).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure was not examined.

In a study investigating effects of TiO_2 NPs on the cardiovascular system, Sprague–Dawley rats (n = 10/sex per group) received by gavage 0, 2, 10 or 50 mg TiO_2 NPs/kg bw per day for 30 or 90 days (5/sex per group terminated at each time point). Body weight was recorded weekly and feed intake every 3–4 days. The heart rate (HR), systolic and diastolic blood pressure (SBP and DBP) were monitored at the start, weekly up to 30 days and every second week thereafter (no information was given about the number of animals per group used for the measurements and whether there were the same animals used repeatedly). At each termination, body and heart weights were recorded and blood (from abdominal aorta) was collected for measurements of activities of creatine kinase (CK), lactate dehydrogenase (LDH) and HBDH as biomarkers of cardiac damage, for clinical chemistry parameters (gluvodr, TGs, TC, HDL-C, LDL-C and absolute leucocyte (WBC) count, TNF-α) and IL-6 as indicators of inflammatory response in the body. Heart tissue samples were taken for histological examination. Treatment with TiO_2 NPs at all doses had no effect on body weight, feed intake or the relative heart weight. Statistically significant changes in heart rate and blood pressure relative to the control group were recorded in mid- and high-dose females as increased DBP on day 47 and decreased SBP on day 57 (no data shown to evaluate a dose response), and mid-dose males as a decreased HR on day 88. The area under curve for SBP was statistically significantly lower for mid-dose females. According to the authors, TiO_2 NPs could induce changes in HR and blood pressure. The Panel noted that each of these transient changes were limited to one sex and considered them not to be treatment related. Statistically significant differences relative to control in biomarkers of cardiac injury were decreased

serum activities of LDH and HBDH in high-dose males in the absence of an apparent dose response relationship and of CK in mid-dose females after 90 days of the treatment. According to the authors, these findings suggested that TiO_2 NPs at high doses could induce cardiac impairment detectable at the level of blood molecular markers. The Panel noted that clinical diagnostic increases in activities of the CK and LDH are regarded as indicative of myocardial injury and increased serum activity of HBDH may reflect renal, red blood cell and/or myocardial damage. No increase in activities of the three biomarkers is in agreement with no histopathological changes in the heart. Values of clinical chemistry parameters were comparable to controls at two terminations with exception of not dose related statistically significantly lower TG in mid- and high-dose males on day 90. Statistically significant increases relative to the control group were reported for total leucocyte count and granulocyte count in high-dose females after 90 days and in total leucocyte count, lymphocyte and monocyte counts in high-dose males after 30 days. The concentration of TNF-α was statistically significantly higher relative to the control in mid-dose females after 30 days. The concentration of IL-6 was statistically significantly increased in mid-dose females and in mid- and high-dose males after 30 days. This, according to the authors, indicated that TiO_2 NPs at high doses could induce inflammatory response in rats. The Panel noted that the changes in WBC, TNF-α and IL-6 were not time- or dose-related. Overall, the Panel considered that gavage administration of TiO_2 NP (24 nm) in doses up to 50 mg/kg bw per day to rats for up to 90 days did not induce any treatment-related effects on the evaluated endpoints.

Grissa et al. (2015)

Test material: TiO_2 NPs, anatase, 5–12 nm (TEM, XRD).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Four-month-old healthy male Wistar rats were provided a commercial pelleted diet and provided drinking water ad libitum. The rats were weighed and randomised into three experimental and one control group (n = 6 per group). Rats were daily administered fresh suspensions of TiO_2 NPs in distilled water by gavage at doses of 0 (distilled water control), 50, 100 or 200 mg/kg bw for 60 days. Body weight changes, feed and water consumption, activity and mortality were monitored daily.

No data on feed consumption, body weight or mortality were reported.

After 60 days, animals were anaesthetised and blood samples were collected by cardiac puncture for haematological parameters, blood smears (and comet assay). Rats were then euthanised. The haematological results indicated statistically significant dose-related decreases in RBC (up to 28%), HCT (up to 23%) and haemoglobin (up to 28%) in exposed animals although the decreases in the last parameter were not dose dependent. Mean corpuscular volume (up to 29%), platelets (up to 42%), mean platelet volume (up to 30%) and WBC (up to 235%) were statistically significantly and dose-dependently increased in exposed animals. The reported data fails to indicate the number of animals used in each investigation. According to the authors, animals exposed to 100 or 200 mg/kg bw TiO_2 NPs (5–12 nm) per day had poikilocytotic (abnormally shaped) hyperchromatic RBCs and abnormally shaped nuclei and hyper-segmented nuclei in lymphocytes and neutrophils.

The Panel considered the changes to be of no toxicological significance.

Grissa et al. (2017)

Test material: TiO_2 NPs, anatase, 5–10 nm (the Panel noted that this material is mentioned as E 171, however, the same material produced by AZ tech (Italy) is described in Grissa et al. (2015) as paint pigment in ceramics).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Four-month-old male Wistar albino rats (n = 6 per group) were randomly divided into control and a TiO_2 NPs (5–10 nm) groups and maintained under control conditions (12-h light cycle, temperature and humidity). The control group received 10 ml/kg bw distilled water and the treated rats received 100 mg TiO_2 NPs/kg bw in suspension in distilled water by gavage, daily for 8 weeks.

During the 8-week treatment, all animals were observed daily for clinical signs and mortality, body weights were evaluated weekly. After 8 weeks and 24 h after the last TiO_2 NPs dose, the animals were weighed and anaesthetised with ether and blood samples were collected by cardiac puncture.

A statistically significantly reduced body weight gain in the treated animal group was observed compared to the control. Serum cholesterol (Chol), glucose (Glu) and TG concentrations were statistically significantly higher in treated rats than in controls (Chol: 2.41 ± 0.11 mmol/L and 1.53 ± 0.02 mmol/L; Glu: 13.37 ± 0.18 mmol/L and 8.18 ± 0.33 mmol/L; TG: 0.69 ± 0.1 mmol/L and 0.34 ± 0.11 mmol/L, treated vs. control).

According to the authors, oxidative stress markers SOD, total antioxidant status (TAS) and catalase (CAT) in plasma were significantly decreased and changes in the lipid peroxidation (LPO) potential were observed in treated rats compare to control. Furthermore, plasma IL-6 was significantly increased in treated rats compare to control.

The Panel noted the changes in glucose levels which are potentially adverse, and considered that the changes in cholesterol and TGs are of unclear toxicological relevance.

Hassanein and El-Amir (2017)

Test material: TiO_2 NPs, 21 nm [no further information].

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Male Sprague–Dawley rats, weighing 160–200 g, maintained under standard environmental conditions, provided standard feed and water ad libitum. After 2 weeks of acclimatisation, the rats were randomly divided into 6 groups (n = 10 rats/group) that received TiO_2 NPs only for 6 weeks. TiO_2 NPs (150 mg/kg bw) in 1% Tween 80 was administered daily by gastric intubation. The control group received 1% Tween 80 (0.5 mL/rat). After 6 weeks of treatment, the animals were terminated and samples from the brain, kidney, liver, lung, heart and testes were stored in 10% buffered formalin for histopathology and prepared for H&E staining. A complete blood count, including erythrocyte count (RBC), haemoglobin (Hb) and total and differential leucocytes (WBC, Lym, Monocy, Neutro), eosinophils (EOS) were performed for each sample. Activities of ALT and AST in the serum samples, serum LPO, total antioxidant capacity (TBAR), glutathione (GSH) and TNF-α were measured by appropriate methods. Furthermore, serum testosterone was determined.

According to the authors, RBC, Hb, WBC, Monocy and EOS were not different compared with control, whereas the Lym and Neutro were statistically significantly increased by treatment with TiO_2 NPs. No units were given for Lym, Monocy and Neutro. Hence, the Panel could not conclude on these data. ALT increased 2.5-fold, AST 2-fold, LPO 2-fold, TNF-α 5-fold, whereas TBAR decreased 2-fold, GSH 4-fold and testosterone 5.5-fold.

In all organs investigated (liver, brain, lung, heart, testis, kidney) 'histopathological' lesions were reported in either 4–6 (++) or 7–10 (+++) rats per treated group, with no lesions found in the control group. The reported findings in the liver were congestion, vacuolar degeneration, mononuclear infiltration in the portal area, focal necrosis with mononuclear infiltration; in the brain: haemorrhage, congestion of choroid plexus blvs, chromatolysis, neuronal degeneration, perivascular lymphocytic cuffing; in the lung: congestion, thrombosis, hyalinisation of the blood vessels wall, hyperplasia of peribronchial lymphoid aggregation; in the heart: vacuolar degeneration and myocardial necrosis; in the testis: congestion and coagulative necrosis; in the kidney: congestion and perivascular mononuclear infiltration. It is not mentioned whether the pathologist performing the histopathology was blinded or whether there was a blinded second reading of the slides. The Panel considered that the descriptions of 'histopathological' lesions are unclear; some of the findings are not histopathological lesions. In addition, the organs in which lesions are reported, the number of lesions per organ and the number of animals with any lesions are not clearly stated.

The Panel noted that only one dose of 150 mg TiO_2 NPs (21 nm)/kg bw has been tested. Based on the many flaws in the study reporting, the Panel was not able to draw any conclusions.

Heo et al. (2020)

Test material: TiO_2 NPs (P25), anatase/rutile, 15–24 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Healthy pathogen-free young adult male and non-pregnant female Sprague–Dawley rats (n = 140) were used. For a repeated-dose 28-day study, animals were randomly divided into four groups (n = 5/sex) following a 6-day acclimatisation period. For the repeated-dose 90-day study, the animals were randomly assigned to four groups (n = 15/sex) following a 6-day acclimatisation period. While 10 animals in each group were terminated for toxicity evaluation after the repeated-dose 90-day study, the remaining 5 animals underwent a 28-day recovery period. No information on the type of diet was provided. The suspensions of TiO_2 NPs (15–24 nm) in sodium phosphate buffer were daily administered in the morning based on the body weight of the animals by gavage TiO_2 NPs at concentrations of 0, 250, 500 and 1,000 mg/kg bw per day with a dosing volume of 10 ml/kg bw.

Food and water intake were recorded before the first administration and then weekly during the study. No statistically significant treatment-related differences with respect to body weight gain, food and water intake were observed. No mortality and clinical signs were detected during the exposure period of 28 and 90 days. No effects were detected in the functional observations battery in the last week of the 90-day study.

Ophthalmoscopic examination and urinalysis did not show statistically significant differences between the groups. Reductions in the number of circulating neutrophils in the 500 mg/kg bw female group and increased numbers of circulating lymphocytes in all three female groups (without a clear dose–response pattern) were reported. These changes were not observed in males. These differences did not persist in the animals in the recovery group and were considered by the authors as spontaneous and not treatment-related. BUN levels were statistically significantly reduced in the 1,000 mg/kg bw female group and blood Na levels were increased in the 1,000 mg/kg bw per day male group.

There were no abnormal gross findings at necropsy in treated animals with or without recovery. Higher absolute pituitary weights in the 1,000 mg/kg bw per day male group, lower absolute uterine weights in the 1,000 mg/kg bw per day female group and higher relative liver weights found in the 1,000 mg/kg bw per day female group. Therefore, the Panel considered these changes as unrelated to the treatment. On histopathological examination, lesions were observed in the kidney, thymus, heart and lung in controls and in the 1,000 mg/kg bw per day dose group in both sexes. The observations did not differ significantly between the control group and the 1,000 mg/kg bw per day group and were not considered to be associated with the treatment by the authors.

The Panel considered that the reported changes were within the historical control range and of no toxicological significance.

Chen et al. (2020a)

Test material: TiO_2 NPs, anatase, 29 nm (STEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Three-week-old healthy male Sprague–Dawley rats were randomly divided into groups of six animals receiving daily by gavage TiO_2 NPs (29 nm) at doses of 0, 2, 10 or 50 mg/kg bw per day for 90 days. Animals were provided a commercial pelleted diet and provided deionised water ad libitum. Behaviour and mortality were monitored daily. Body weights were assessed every 7 days and at termination, while the food intake was recorded every 3–4 days. Blood from the abdominal aorta was taken at termination. In addition, SCFAs in stool samples were analysed by targeted metabolomics using gas chromatography–tandem mass spectrometry (GC–MS/MS).

No mortality was observed during the study. Starting from week 8, the 10 and 50 mg/kg bw per day groups showed decreased body weight gains up to about 15%, while food intake was not different between the groups

Serum levels of triglycerides in the 10 and 50 mg/kg bw per day groups were statistically significantly lower than in the control group while serum TC, HDL-C and LDL-C were not affected. In an untargeted metabolomic analysis, 343 of 1,837 lipophilic metabolites were differentially expressed between controls and the 50 mg/kg bw per day group.

No statistically significant differences in organ weights for the heart, spleen, liver, kidney, lung, stomach and testis were observed. No further results were reported.

Six SCFAs in faeces, including acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and hexanoic acid, did not change after exposure to TiO_2 NPs.

The Panel considered that, while the change in body weight gain may be adverse, other reported changes were of no toxicological significance.

Chen et al. (2020b)

Test material: TiO_2 NPs, anatase 24 nm (SEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Three-week-old Sprague–Dawley rats were fed a commercial pellet diet and deionised water ad libitum. After 1 week of acclimation, rats were weighed and randomly divided into experimental and control groups. Rats (n = 5/sex per group) were daily administered by gavage with 0, 2, 10 or 50 mg/kg bw per day with TiO_2 NPs (24 nm) with or without 1.8 g/kg bw glucose in 1 mL of water for 90 consecutive days.

Body weights were determined weekly and there were no significant differences in body weight gains in any group during the study.

After fasting overnight, blood was drawn from the tail vein of rats at 9, 16, 23, 30, 44, 58, 72 and 90 days to monitor blood glucose levels. Although differences were seen in treated and control rats, these were only in female rats and at occasional time points. The authors calculated the area under the curve (AUC) of blood glucose and determined that the female rats treated with glucose alone and female rats treated with glucose with either 2 or 10 mg/kg bw per day TiO_2 NPs had slight but significantly reduced AUCs. After 90 days, animals were weighed and killed. Blood samples were collected from the abdominal aorta and the levels of blood glycoproteins, including glycated haemoglobin (HbA1c) and glycated serum protein (GSP) were also measured. Rats treated with glucose and TiO_2 NPs (10 and 50 mg/kg bw per day groups) had a significantly reduced levels of HbA1c in female rats. Male rats treated with glucose and TiO_2 NPs (2 mg/kg bw group) had a significantly reduced level of GSP.

Blood insulin, C-peptide and glucagon levels were also determined at a single undeclared time point in the study by a radioimmunoassay. Considering only those effects in which there appeared to be a potential dose–response effect of TiO_2 NPs (no dose response effects were seen when glucose was co-administered with TiO_2 NPs), blood insulin levels were statistically significantly lower than control in females in the 10 or 50 mg/kg bw per day TiO_2 NPs groups, but there was no effect in males. In males, C-peptide was significantly lower in the 50 mg/kg bw per day TiO_2 NPs group, but no such effect was seen in females. No clear dose–response effect was seen on glucagon levels.

Prior to termination, an OGTT was performed at day 90. The rats were fasted overnight for 16 h and subsequently challenged with glucose alone at 2 g/kg bw via gavage. Blood glucose levels were determined at 0 h (pre-glucose treatment) and at 30, 60, 90 and 120 min (post-glucose treatment). Although differences were seen in blood glucose concentrations, these were only in male rats (at 30 and 60 min after glucose challenge in the 2 mg/kg bw per day TiO_2 NPs group and at 60 min after glucose challenge in the 50 mg/kg bw per day TiO_2 NPs + glucose group). Only rats treated chronically with both glucose and 50 mg/kg bw per day TiO_2 NPs showed a significant increase in AUC for blood glucose in the glucose tolerance tests.

Pancreata were excised, fixed and processed for histopathological examination by a pathologist who was blinded to the treatment group and dosing regimen. No pathological changes associated with TiO_2 NP administration were observed.

In the absence of any clear dose–response effects for TiO_2 NPs (24 nm) and the occasional nature of these changes, the Panel considered the changes in blood glucose, HbA1c, GSP, insulin, C-peptide, glucagon and glucose tolerance as either not test substance related or irrelevant for the safety evaluation of TiO_2 NPs.

Reproductive and developmental toxicity studies

TiO_2 *NPs or* TiO_2 *containing a fraction of nanoparticles*

Rats

Warheit et al. (2015b)

In this study, there were five different test materials relevant for E 171: 1) anatase/rutile (89/11%) (uf-1), d_{50} = 43 nm (XSDC), d_{50} = 23 nm (TEM), irregular; 2) anatase (100% nano) (uf-2),

d_{50} = 42 nm (XSDC), d_{50} = 19 nm (TEM), irregular; 3) rutile (100% nano) (uf-3), d_{50} = 47 nm (XSDC), d_{50} = 22 nm (TEM), rod-like; 4) anatase (27% nano) (pg-1), d_{50} = 153 nm (XSDC), d_{50} = 120 nm (TEM), irregular; 5) rutile (11% nano) (pg-2), d_{50} = 195 nm (XSDC), d_{50} = 165 nm (TEM), irregular. The purity of each tested material was not reported but the most abundant metals were analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

These studies report prenatal developmental toxicity studies with different tested materials in pregnant rats, performed in accordance with OECD TG 414. The test substances were formulated in sterile water. Dosing formulations were collected and analysed near the beginning and end of the dosing period for analyses. These analyses confirmed that the formulations were at the targeted concentrations, were uniformly mixed and were stable under the experimental conditions used during the study.

In three studies, time-mated pregnant Sprague–Dawley, Crl:CD(SD), rats (n = 22/group) were daily exposed to TiO_2 (uf-1, uf-3 and pg-1) by gavage on GDs 6–20. In three additional studies, pregnant Wistar rats (n = 22–23/group) were daily exposed to TiO_2 (uf-2 and pg-2) by gavage from GDs 5 to 19. The dose levels used in the studies were 0, 100, 300 or 1,000 mg/kg bw per day. The dose volume was 5 mL/kg bw per day. Clinical signs were recorded at least daily. Body weight and feed intake were measured at regular intervals. Sprague–Dawley rats were killed for a caesarean section on GD 21 and Wistar rats on GD 20. Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to identify any abnormalities. At 1,000 mg uf-1/kg per day, mean fetal sex ratio and the means for male and female fetuses per litter were statistically significantly different from the control group means. The mean number of male fetuses was 7.2 compared with 5.5 male fetuses for the concurrent control group; the test facility historical control group data ranged at that time from 5.2 to 7.4. The mean number of female fetuses was 4.8 compared with 6.7 for the concurrent control group; the test facility historical control group data ranged at that time from 5.8 to 8.3. Mean fetal sex ratio of the 1,000 mg uf-1/kg bw per day group was 60% (males/females) compared with a sex ratio of 46% in the concurrent control group; the test facility historical control group data ranged at that time from 43% to 53%. Apart from some incidental changes in body weight and feed intake, no other changes were observed in the dams or the fetuses in these studies. The authors concluded that there were no significant toxicological or developmental effects in females or fetuses at any of the dose levels or compounds tested and considered the NOAEL for each compound to be 1,000 mg/kg bw per day, the highest dose tested.

The ANS Panel (EFSA ANS Panel, 2016) agreed with the authors. The Panel agreed with both the author and ANS Panel conclusions.

TiO_2 NPs < 30 nm

Mice

Khorsandi et al. (2016)

Test material: TiO_2 NPs (no further information, the Panel assumed that the test material is the same as in Khorsandi et al., 2017).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

The study examined the effects of oral administration of TiO_2 NPs on testis volume, seminiferous tubules, interstitial tissue and total Leydig cell numbers in male NMRI mice that were 6- to 8-week-old at the start of the study. TiO_2 NPs dispersed in BSA solution were administered by oral gavage at doses of 0, 75, 100 and 300 mg/kg bw per day to randomly selected groups of 8 animals for 35 days. The authors did not provide data on the general toxicity of the treatment nor for the systemic absorption of the test substance. One day after the last administration blood samples for the determination of circulating testosterone were taken and the mice sacrificed by cervical dislocation

under ether anaesthesia. Endpoints included body and testis weight and volume at termination, testicular testosterone concentration and testicular histology and morphometry after fixation in Bouin's solution. While body weight was unaffected by treatment, the authors reported dose-dependent decreases in testis weight from a dose of 100 mg/kg bw per day. The Panel noted that there is a discrepancy between the statement in the text that a statistically significant decrease was only observed at 300 mg/kg bw per day, and the presentation in Table 1 of the publication that indicates significant differences to the control in both the mid- and high-dose groups. These groups also showed decreases in serum and testicular testosterone levels, the diameter and total volume of seminiferous tubules, the height of the spermatogenic epithelium and total Leydig cell numbers. Contrarily, the total volume of the interstitial tissue was found to be increased.

The Panel considered that TiO_2 NPs (size unknown) at 100 mg/kg bw per day had an effect on testis weight.

Khorsandi et al. (2017)

Test material: TiO_2 NPs, 20–30 nm (AFM), crystalline form unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

This single dose level study examined the effects of quercetin on the TiO_2-induced damage on reproductive parameters in male NMRI mice. In this assessment, only the results from the TiO_2 NPs and the control groups are discussed. TiO_2 NPs were dispersed in BSA solution and administered by oral gavage at a dose of 300 mg/kg bw per day to a randomly selected group of 8 animals for 35 days. Treatment was started after the animals had been given oral saline for 7 days. Controls received saline throughout. The authors did not provide data on the general toxicity of the treatment nor for the systemic absorption of the test substance. One day after the last administration, blood samples for the determination of circulating testosterone were taken and the mice sacrificed by cervical dislocation under ether anaesthesia. Endpoints included body and testis weight, testicular testosterone, MDA, SOD and CAT concentrations, testicular histology and apoptosis assessment by a TUNEL assay after fixation in Bouin's solution, as well as cauda epididymis sperm counts, motility and morphology. The authors reported significant decreases in testis weight, circulating and testicular testosterone, testicular CAT and SOD concentrations, sperm counts and sperm motility. Significant increases were found in the percentage of abnormal or degenerative spermatogenic tubules, germ cell apoptosis, testicular MDA concentration and in the percentage of sperm with abnormal morphology. Body weight in the TiO_2 NPs group was similar to the control at termination.

The Panel considered that testicular toxicity was observed with TiO_2 NPs (20–30 nm) at a dose of 300 mg/kg bw per day, the only dose tested.

Karimipour et al. (2018)

Test material: TiO_2 NPs, anatase, 10–25 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

The study examined the effects of oral administration of TiO_2 NPs for 5 weeks on the histology of ovaries, oestrogen and MDA serum levels (7 animals/group), fertility (10 animals/group) and IVF rates (10 animals/group) in female mice (presumably NMRI). TiO_2 NPs dispersed in phosphate buffered saline with 0.5% Tween 80 were administered by gavage at a dose of 100 mg/kg bw per day. Mice were randomly assigned to the control and treatment group. General endpoints for toxicity such as body weight development, food consumption, clinical signs and systemic absorption of TiO_2 were not examined/reported. The authors reported a significantly decreased pregnancy rate (70% vs. 100% in the control group), a 20% decrease in litter size and increases in circulating oestrogen (20%) as well as MDA (25%). They observed degeneration and reduction of follicles, cyst formation and impairment of follicular development in the ovaries of the TiO_2 NPs group but presented no quantitative data. The *in vivo* findings were supported by a lower number of oocytes isolated from the exposed group and a higher percentage of developmental arrest before the blastocyst stage after *in vitro* fertilisation. The

authors suggest that the observed effects could be the consequence of an indirect effect of TiO_2 NPs through the generation of increased ROS levels.

The Panel considered that the study shows an impairment of female fertility at a dose of 100 mg TiO_2 NPs (10–25 nm)/kg bw per day.

Karimi et al. (2019)

Test material: TiO_2 NPs, 68 nm (DLS; no direct information on constituent particle size, the Panel considered the majority of constituent particles to be below 30 nm), crystalline form and purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Four groups of eight 6- to 8-week-old male NMRI mice were daily treated by gavage with either (1) saline for 42 days, (2) 200 mg/kg bw per day curcumin for 42 days, (3) with saline for 7 days followed by 50 mg TiO_2 NPs/kg bw per day for 35 days or (4) the simultaneous combination of exposures to curcumin and TiO_2 NPs as in (2) and (3). In this assessment, only the results from the TiO_2 NPs and control groups are presented Testicular damage was studied. The authors do not report on general health or clinical signs, nor is there data showing systemic absorption of TiO_2. TiO_2 NPs at 50 mg/kg significantly reduced testis weight in the presence of a non-significant trend towards lower body weight, accompanied by reduced serum testosterone to around 30% of control levels. Similarly, seminiferous tubule diameter and epithelium height were affected by TiO_2 NPs treatment. TiO_2 NPs treatment significantly reduced the maturity of the germinal epithelium, as determined by the Johnsen's scoring system. Similar significant adverse findings were observed in reduced sperm counts, increased sperm abnormalities and reduced sperm motility.

The Panel noted that 50 mg TiO_2 NPs (< 30 nm)/kg bw per day resulted in adverse effects on the testis.

Lu et al. (2020)

Test material: TiO_2 NPs, anatase, 7 nm (TEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 3.

Internal exposure not examined.

Four groups of 15 male ICR mice, age 6–8 weeks, were daily treated by gavage with TiO_2 NPs at doses of 0, 10, 50 or 100 mg/kg bw per day for 30 days.

Animals were fasted for 10 h before each administration. Effects were studied on the blood–testis barrier (BTB), MAPK signalling pathways, serum testosterone and oestradiol levels and sperm parameters. The authors did not report on general health and clinical signs, nor is there data showing systemic absorption of TiO_2. Through TEM, the authors report tight junction damage in the BTB at 50 and 100 mg/kg bw per day, though the histopathological pictures provided are hard to interpret. BTB-related proteins F-actin, ZO-1 and claudin-11 were dose-relatedly significantly increased up to 2-fold at the high dose, with no significant changes observed in connexin-43 and occludin. F-Actin was significantly increased at all doses tested. As to elements of MAPK signalling pathways, ERK and JNK mRNA expression was slightly but significantly increased in testis tissue at the high dose only. No effect was found on p38 mRNA expression. Serum testosterone was 50% decreased at the two highest doses tested, accompanied by a slight but significant reduction in oestradiol at the same doses. Sperm counts were unaffected by exposure, but sperm motility was dose-relatedly reduced from around 70% in controls to around 50% at the high dose, accompanied by increased sperm malformation rates from 3% in controls to 8% at the high dose, both effects being statistically significant at the two highest doses.

The Panel considered that TiO_2 NPs (7 nm), at 50 or 100 mg/kg bw per day, resulted in a dose-related reduction of sperm motility and increased sperm malformations, accompanied by histological observations in the testis, changes in BTB-related protein levels, changes in MAPK-related mRNA levels and reduced circulating testosterone concentrations.

Rats

Lee et al. (2019)

Test material: TiO_2 NPs (the Panel noted that from the description of the tested material, it corresponds to P25 (15–24 nm)).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure examined: quantitative analysis in blood/tissues; methodology reliable with some limitations.

Mated Sprague–Dawley rats (12 females per group) were daily administered by gavage TiO_2 NPs at dose levels of 0, 100, 300 and 1,000 mg/kg bw per day from GD 6 to 19. The study was designed in accordance to OECD Guideline 414 and in compliance with GLP. The dose volume was 10 mL/kg bw per day. Clinical signs were recorded at least daily. Body weight and feed intake were measured at regular intervals. Rats were killed for a caesarean section on GD 20. Gross necropsy included gross examination of the dam, counting of the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, fetal sex and weight. Fetal pathological external, visceral and skeletal examinations were performed in order to detect abnormalities. In addition, the following organs were weighed and the absolute and relative organ weights were presented: adrenal glands, brain, heart, kidney liver, pituitary gland, spleen, ovaries (right, left) and thymus.

There were no statistically significant differences in general clinical signs, body weight, organ weights (absolute and relative to body weight), macroscopic findings, apart from a statistically significant decrease in food intake of the females of the high-dose group. The authors considered that this decrease did not have toxicological significance since it was minimal and there was no correlated decreased body weight or body weight gain during the study period. The Panel agreed with this conclusion.

Caesarean section parameters and fetal external and visceral examinations did not reveal any statistically significant differences. The only difference seen was a small but statistically significant increase (4%) in the number of ossification centres in the metatarsals of both hindlimbs of the fetuses of 100 mg/kg bw per day group. The Panel agreed with the authors that this could be considered as an incidental finding as no other related effects were observed.

The Panel considered that no adverse effects were reported with TiO_2 NPs (21 nm) up to 1,000 mg/kg bw per day, the highest dose tested.

Neurotoxicity and neurodevelopmental toxicity studies

TiO_2 NPs

Rats

Ebrahimzadeh et al. (2017)

Test material: TiO_2 NPs, anatase, < 100 nm (TEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Pregnant Wistar rats (n = 6/group, randomly assigned) were administered by gavage TiO_2 NPs in distilled water vehicle (volume not reported) at doses of 0 or 100 mg/kg bw per day from GD 2 to 21 (gestation group) or from PND 2 to 21 (lactation group).

Endpoints were quantification of hippocampal apoptosis (TUNEL, RT-PCR) and neurogenesis (DCX-positive cells) in CA1-3 and dentate gyrus (DG) on PND 1 (gestation group) or PND 21 (lactation group) (n = 6: 1 male/litter, 6 litters/group; unclear if TUNEL and DCX n = 3/group).

Both gestational and lactational exposure significantly increased hippocampal apoptosis in CA1-3 and DG, and reduced neurogenesis in CA1-3.

The Panel agreed with the author's conclusion that exposure with TiO_2 NPs at 100 mg/kg bw per day during pregnancy and lactation increased apoptosis and reduced neurogenesis in the hippocampus of the offspring.

Kandeil et al. (2019)

Test material: TiO_2 NPs, 90 nm (range 40–140 nm) (TEM), crystalline form and purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Adult male albino rats (n = 20/group, randomly distributed) were administered by gavage TiO_2 NPs in distilled water (volume not reported) at doses of 0 or 500 mg/kg bw per day for 14 days.

The main endpoints examined were clinical signs, brain homogenate dopamine and 5-hydroxytryptamine, cholinesterase activity, oxidative stress markers (GSH, SOD, MDA, total antioxidant capacity (TAC), TOS, OSI = TOS/TAC), inflammatory and apoptosis markers (IL-1β, TNF-α, caspase-3, Fas; rf2, N N QO1 and INOS gene expression), cerebral mitochondrial viability (MTT), cerebral DNA fragmentation (diphenylamine colorimetric) and prefrontal cortex histopathology (blinded quantitative).

The TiO_2 NPs group showed decreased physical activity, passive behaviour, loss of appetite and tremors. Treatment statistically significantly reduced brain GSH, SOD, TAC, Nrf2 and NQO1 expression; significantly reduced cerebral mitochondrial viability; increased DA and 5-HT; increased cholinesterase activity; increased MDA, TOS and OSI levels; increased IL-1β, TNF-α, caspase-3, Fas; INOS expression; increased cerebral DNA fragmentation; and prefrontal cortical congestion, pericellular oedema, perivascular oedema and pyknosis.

The Panel considered that these data show that oral TiO_2 NPs can induce CNS toxicity at 500 mg/kg bw per day, possibly related to oxidative stress.

TiO_2 NPs < 30 nm

Mice

Zhou et al. (2017)

Test material: TiO_2 NPs, anatase, 6–7 nm (TEM, XRD, Hu et al., 2011).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 3.

Internal exposure: quantitative measured in tissues; methodology not reliable.

Pregnant or lactating CD-1 (ICR) mice (n = 6/group, randomly assigned) were administered by gavage TiO_2 NPs in 0.5% w/v aqueous hydroxypropylmethylcellulose vehicle (volume not reported) at doses of 0, 1, 2 or 3 mg/kg bw per day from 'prenatal day 7', presumed to be GD 7, although possibly GD 14, to PND 21.

Endpoints in primary cultures of hippocampal CA1 neurons harvested after PND 21 from the pups were: neuron ultrastructure (by TEM), dendrite morphology (by SEM), mitochondrial membrane potential (MMP; fluorescence), ROS, MDA, protein carbonylation (PC), ATP levels, apoptosis- and autophagy-related factors (ELISA, western blot) (n = 5/group per endpoint).

The Panel noted that the method applied to randomise the pups from 6 dams/group into 30 pups/group is not reported. It is also unclear whether CA1 neurons were harvested from all 30 pups/group.

The authors reported that the Ti amount in hippocampus was dose-dependently increased at all doses. The Panel noted that the description of the analytical method was insufficient to establish its reliability and tissue concentrations were expressed with incorrect measurement units.

At all doses, there were dose-dependent decreases in dendritic growth (primary dendrite length, significantly reduced by 24.71%, 63.82% and 77.99% at low, mid and high doses, respectively), MMP (significantly reduced by 17.14%, 36.57% and 51.43% at low, mid and high doses), and concentrations of MDA, PC and ATP (nmol/mg protein).

At all doses, apoptosis- and autophagy-related factors (rH2AX, Cytc, caspase 3, PI3K3C, Beclin 1 and c-Jun; LC3I, LC3II, JNK and p-JNK) were significantly and dose-relatedly increased, and anti-apoptosis-related protein Bcl-2 was significantly and dose-relatedly decreased.

Tissue ROS was significantly increased at mid and high doses only (to the same level, no dose response).

Treatment-related neuronal ultrastructural changes were reported (narrative only, no quantitative data), including mitochondrial swelling, carina disappearance, nucleus shrinkage, chromatin marginalisation, anomalous nuclear membrane and dilation of endoplasmic reticulum accompanied by the emergence of autolysosomes and the formation of vacuoles. The Panel noted that TiO_2 NPs (6–7

nm), at all doses tested, inhibited dendritic outgrowth, reduced mitochondrial function and increased autophagy and oxidative stress markers, in *ex vivo* hippocampal CA1 neurons after dosing during gestation and early lactation.

Zhang et al. (2020)

Test material: TiO_2 NPs, 21 nm (TEM), crystalline form and purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Young adult (age 7 weeks) male C57BL/6J mice (n = 30 randomised into two groups; presumably n = 15/group but not reported) were administered by gavage TiO_2 NPs in vehicle (2% heat-inactivated mouse serum, volume not reported) at doses of 0 or 150 mg/kg bw per day for 30 days.

Main endpoints were small intestine and brain (hippocampus and cerebral cortex) histopathology, gut microbiota (faecal bacterial 16S rRNA gene sequencing), gut and cerebral cortex transcriptomics and functional tests (open field test (OFT), Morris water maze (MWM)). It is noted that the number of animal tested is not reported, but results for some endpoints show n = 10/group.

Treatment had no effect on body weight or histopathology of gut or brain, but significantly decreased the richness and evenness of gut microbiota (decreased Shannon's diversity, chao, observed species and elevated Simpson's diversity) and elevated gut HuC/D and TuJ1, suggesting an effect on the enteric nervous system.

Serotonergic markers Sstr1 and Sstr2 were markedly reduced in the gut but not in the cerebral cortex. Gut–brain peptides secreted by endocrine cells and enteric neurons, and also inflammatory cytokines, were not affected by treatment.

OFT centre field activity was markedly reduced by treatment, consistent with anxiety-like behaviour, but MWM learning and memory were unaffected.

The Panel considered that these data show that TiO_2 NPs (21 nm) can markedly alter the mouse gut microbiota, without pathological changes in small intestine and brain. Spatial learning and memory were not affected but centre field activity in open field testing was decreased at the only dose tested of 150 mg/kg bw per day, consistent with increased anxiety. The Panel is aware of ongoing research on relationships between gut microbiota and anxiety (Yang et al., 2019)

Rahnama et al. (2020)

Test material: TiO_2 NPs, 21 nm (TEM), crystalline form and purity unknown.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 3.

Internal exposure not examined.

Effects of TiO_2 NPs were investigated on stereological parameters (volume of brain region and number of neurons) in the hippocampal DG and on the qualitative morphology of hippocampal granular neurons in adult mice.

Adult male mice (n = 20, 10–12 weeks old), food and water ad libitum, were randomly divided over four groups (no further information reported) and administered daily by gavage a suspension of TiO_2 NPs at doses of 0 (saline control), 2.5, 5 or 10 mg/kg bw per day for 35 days.

After formalin perfusion fixation, brains were removed and the two hemispheres carefully divided. One hemisphere (chosen randomly) was used for stereology; the other for qualitative morphological examination of volume appearance of the hippocampus, DG and sublayers (H&E systematic sections; and silver Golgi staining for neuronal dendrite appearances (dendritic length and branching). The total volume of the hippocampus, DG (including also the different sublayers) was estimated using the Cavalieri principle and H&E-stained systematically sampled sections. The numerical density of DG granular cells was estimated with physical dissector. The morphology of DG granular cells was studied using qualitative silver nitrate Golgi staining. The Panel noted that in the Methods section, the correct formula for volume estimation according to the Cavalieri principle is missing; instead, the formula for numerical density estimation using physical dissector is erroneously given twice.

Compared to the saline control group, TiO_2 NPs induced dose-related reductions in the total volume of the hippocampus (statistically significant in the low-, mid- and high-dose groups), of the DG (statistically significant in the mid- and high-dose groups), including the volume of its molecular and

granular layers (both statistically significant in the mid- and high-dose groups) and the volume of the polymorph layer (statistically significant in the low-, mid- and high-dose groups). In addition, there was a dose-related reduction in the numerical density of DG granular cells, as well as a dose-related reduction in the total number of granular cells of the DG (both statistically significant for the low-, mid- and high-dose groups).

According to the authors, qualitative examination of the morphology of the hippocampus and DG granular cells supported the measured reduction in volume of hippocampus and DG and suggested that the length of the dendrites of the granular cells appeared shorter and the number of branches reduced. The Panel noted that in Figure 9, photographs of five groups are shown (instead of four), illustrating the morphological appearances of the hippocampus/DG. The Panel noted that the results show both a control and a sham without describing these controls in the methods.

The Panel agreed with the authors that the results of the (unbiased) stereological data will contribute to the understanding of memory and learning disorders resulting from exposure to TiO_2 NPs.

The Panel noted that TiO_2 NPs (21 nm) at all doses tested lead to a reduced volume of the hippocampus, and the polymorph layer of the DG, and on reduced numerical density and total number of granular cells of the DG.

Rats

Grissa et al. (2016)

Test material: TiO_2 NPs, anatase, 5–10 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Adult male Wistar rats (n = 6/group, randomly assigned) were administered by gavage TiO_2 NPs at doses of 0, 50, 100 or 200 mg/kg bw per day in 10 mL water vehicle/kg bw for 60 days.

The main study endpoints were necropsy body and brain weight, plasma and brain IL-6, whole brain homogenate cholinesterase activity, cerebral cortex GFAP-positive cells (by immunohistochemistry, counted in 'areas that had a maximum of positive cells', n = 3/group)

Body weights were unaffected by treatment, but relative brain weight was dose-dependently decreased, significantly at 100 and 200 mg/kg bw per day. Plasma IL-6 was increased at all doses (no dose response). Brain IL-6 was dose-dependently increased significantly at 100 and 200 mg/kg bw per day. Plasma cholinesterase activity was statistically significantly and dose-dependently reduced at all doses (by about 35%, 50% and 50% at 50, 100 and 200 mg/kg bw per day, respectively). The Panel noted the methodology of the authors did not indicate whether plasma cholinesterase activity represented acetylcholinesterase or butyrylcholinesterase or both. Brain cholinesterase activity was reduced at 100 and 200 mg/kg bw per day only (at both doses by about 50%, i.e. no dose response). Cerebral cortex GFAP-positive cell counts were dose-dependently increased at 100 and 200 mg/kg bw per day. The Panel noted that TiO_2 NPs (5–10 nm) reduced plasma cholinesterase activity and increased plasma IL-6 at all dose tested levels, and reduced brain cholinesterase activity at doses of 100 and 200 mg/kg bw per day. The Panel noted reduced plasma cholinesterase activity and increased plasma IL-6 at all doses and reduced brain cholinesterase activity at 100 mg/kg bw per day.

Mohammadipour et al. (2016)

Test material: TiO_2 NPs, anatase, 10 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Wistar rat dams (n = 6/group, randomisation not reported) were administered by gavage TiO_2 NPs in distilled water (volume not reported) at doses of 0 or 100 mg/kg bw per day.

To avoid possible dermal and oral transfer, the animals' cages were cleaned daily. Thus, according to the authors, the exclusive and significant way of offspring exposure to TiO_2 nanoparticles was through maternal milk.

Offspring were weaned and housed 5/cage from PND 21, and learning/memory tested from PND 60.

Endpoints were learning and spatial recognition memory in Morris water maze (MWM) and two-compartment light/dark shock passive avoidance (PA) tests.

MWM acquisition path length and latency were initially higher in the treated group on days 1–3, but normal on days 4–5. Performance in the memory probe trial was unaffected by treatment.

In the PA test, latency to enter the dark compartment was significantly lower in the treated than the control group at 1 and 24 but not 48 h post-shock; total time spent in the dark compartment was significantly higher than in controls at all three time points post-shock.

The authors concluded that TiO_2 NPs dosing of lactating mothers impairs memory and learning of pups in adulthood. However, the Panel noted that MWM acquisition (days 4 and 5) and retention probe trial) were normal, and the reduced latency to dark compartment entry post-shock was transient (1 and 12, not 48 h). The Panel considered that these data do not show that maternal exposure to TiO_2 NPs (10 nm) during lactation impairs learning and memory in the offspring.

Hassanein and El-Amir (2017)

Test material: TiO_2 NPs, 21 nm [no further information].

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 4.

Internal exposure not examined.

Adult male Sprague–Dawley rats (n = 10/group, randomly assigned) were daily administered by gavage TiO_2 NPs in 1% Tween 80 (0.5 mL/rat) at doses of 0 or 150 mg/kg bw per day for 6 weeks.

The only reported endpoint relevant to neurotoxicity was brain histopathology.

Reported findings were haemorrhage, congestion of choroid plexus 'blvs' (the Panel interpreted this as blood vessels), chromatolysis, neuronal degeneration and perivascular lymphocytic cuffing, each in at least 4 of 10 treated animals.

The Panel noted that effects on brain histopathology were observed at a dose of 150 mg/kg bw per day TiO_2 NPs (21 nm), the only dose tested, although the tissue fixation method was not optimal for histology of brain tissue (non-perfused formaldehyde fixation).

Canli et al. (2020)

Test material: TiO_2 NPs, anatase, 21 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 3.

Internal exposure: qualitative analysis in tissues; methodology with important flaws.

Adult female albino rats (n = 6/group, randomisation not reported) were administered by gavage TiO_2 NPs at doses of 0, 0.5, 5 or 50 mg/kg bw per day for 14 days.

Main endpoints were liver thiobarbituric acid reactive substances (TBARS) and GSH, ATPase activity in supernatant of homogenised kidney, intestine and brain, and cholinesterase activity in brain homogenate supernatant. TEM images of TiO_2 NP in liver, kidney and brain were recorded. Body and organ weights were not reported.

The authors reported that TEM demonstrated the presence of TiO_2 particles in the liver, kidney and brain which 'seemed dose dependent'). The Panel noted that verification of the elemental composition of the particles of interest was not performed. There was one death at 0.5 mg/kg bw per day group (no further details reported), but no other notable clinical signs (e.g. 'eye colour, feeding habits, activity'). Treatment had no effect on liver total, reduced or oxidised glutathione (tGSH, rGSH or GSSG) or the ratio between reduced and oxidised glutathione (GSH/GSSG ratio), or on kidney and intestine ATPase activity. Brain Na/K-ATPase activity was significantly increased (approximately 2-fold) at 0.5 and 5 mg/kg bw per day, Mg-ATPase and total ATPase activity at 5 mg/kg bw per day. Brain cholinesterase activity was significantly reduced at all doses (by about 50%, 35% and 50% at 0.5, 5 and 50 mg/kg bw per day, respectively, i.e. no dose response).

The Panel noted that reduced brain cholinesterase activity was observed at all dose levels tested, while increased brain Na/K-ATPase activity was observed only at the lowest dose tested of 0.5 mg/kg bw per day and at the highest dose tested of 50 mg/kg bw per day of TiO_2 NPs (21 nm).

Grissa et al. (2020)

Test material: TiO_2 NPs, anatase, 5–12 nm (TEM, XRD).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure: quantitative in tissues; methodology with important flaws.

Male Wistar rats were administered by gavage (aqueous suspension; volume of 10 mL/kg bw) with TiO_2 NPs (five times/week; 8 weeks) after being randomised over 4 groups (n = 8/group): 0 (control), 50, 100 and 200 mg TiO_2 NPs/kg bw per day. Clinical signs and mortality were recorded daily. Rats were euthanised the day after treatment. The brain was removed and weighed and frontal lobes isolated (right one was formalin-fixed; part of left homogenised). Titanium amount was quantified in the homogenate.

Brain frontal lobe histopathology, biomarkers of oxidative stress (antioxidant enzyme activity (SOD), glutathione peroxidase (GPx), TAS, CAT, TBARS and inflammatory markers nitric oxide (NO) and TNF-α) were examined.

The amount of titanium in brain after intragastric administration of TiO_2 NPs for 8 weeks was significantly and dose-dependently elevated in all dose groups compared to controls (in which no titanium was detected). However, the Panel noted that the measured Ti levels in tissues were below the LOQ.

Brain histopathology was reported at mid and high doses (qualitative description only): lymphoid infiltration, probably due to cerebral inflammation and hypersensitivity, vascular congestion, cerebral oedema, proliferation of glial cells, some cell necrosis and neuron cells cleavaged into filamentous shapes. At low dose, authors reported 'quasi-normal histological structure, suggesting no abnormal pathological changes in the cerebral cortex'.

SOD and CAT activities were significantly decreased at all doses, and GPx activity and total antioxidative status (TAS) at mid and high doses. Inflammatory biomarkers: NO was significantly increased at mid and high dose, and TNF-α at high dose. Lipid peroxidation: TBARS was significantly increased at mid and high doses. Apoptosis (by TUNEL) was significantly increased at the high dose.

The Panel noted that reduced activity of brain frontal lobe SOD and CAT were observed at all doses of TiO_2 NP (5–12 nm).

Inflammation and immunotoxicity studies

E 171

Mice

Urrutia-Ortega et al. (2016)

Test material: E 171, particles below and above 100 nm, crystalline form not reported.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure: qualitative analysis in tissues, methodology reliable with some limitations.

BALB/c male mice of 4- to 6-week-old after 1 week of acclimation were randomly divided in to four groups (n = 6): a) control, b) E 171 group, c) chemically colitis-associated colorectal cancer (CAC) group and d) CAC + E 171 group. The E 171 group of mice received an administration by gavage of 5 mg/kg bw per day for 5 days/week during 10 weeks. The CAC group received a single intraperitoneal dose of 12.5 mg/kg bw azoxymethane (AOM) and 2% dextran sulfate sodium (DSS) in the third, sixth and ninth week in water ad libitum. The CAC + E 171 group received a single dose of AOM and DSS in the same scheme of CAC group, and in addition, received administration of E 171 by gavage according the same scheme as the E 171 group. The control group received a single intraperitoneal injection of saline solution. After 11 weeks, mice were sacrificed and the colon, kidney, liver, spleen, lung tissue and blood samples collected. The colon was dissected, opened and fixed in paraformaldehyde. For histological examination, sections were stained with H&E (blinding is not mentioned). Tumour progression was evaluated by the detection of COX2, β-catenin and Ki67, while NF-κB was used as a key marker of inflammation in colon tissue by immunohistochemistry. Goblet cells were identified by counting Alcian blue-stained blue cells. In colon homogenates tissue levels of IL-2, TNF-α, IFN-γ, IL-10 and GM-CSF were determined by Bio-Plex multiplex MAGPIX.

Mice body weight and consumption of food and water remained unchanged by administration to E 171 by gavage. Regarding organ weights, the spleen, liver and lung weights remained unchanged, with no histological abnormalities in these organs. Mice from group CAC + E 171 group had a decrease of 74% in kidney weight; however, E 171 administration had no effect on this parameter. BUN and serum creatinine, used as kidney function markers, however, remained without changes (data not shown).

Regarding tumour formation and inflammation, this study aimed to investigate the impact of oral E 171 intake on the enhancement of colorectal tumour formation in a CAC mouse model. Results obtained indicate that E 171 alone was unable to induce tumour formation, but dysplastic alterations were observed in the distal colon with a statistical significant enhancement of tumour formation in CAC + E 171 group vs. CAC group ($p < 0.01$). Some E 171 particles were internalised in colonic cells of the E 171 and CAC + E 171 groups, and both groups showed a decrease in goblet cells in the distal colon. CAC tumour progression markers including COX2, Ki67 and b-catenin indicated that E 171 exacerbates tumour progression and p65 NF-κB, a key regulator of inflammation was also induced by E 171 administration in CAC group. In the E 171 group, despite the absence of tumour formation, a slight statistical significant increase in COX2, Ki67 and b-catenin, and p65 NF-κB were observed. Regarding cytokines in colon tissue, no changes, despite enhanced the p65-NF-κB expression, were found in the E 171 alone and CAC groups. Furthermore, a statistically significant decreases of IL-2, TNF-α, INF-g and IL-10 were observed in the CAC + E 171 group when compared to the E 171 group, probably as a consequence of colon tissue damage.

The Panel considered that the results suggest that while E 171 (5 mg/kg bw per day) alone administered for 10 weeks has no effect on tumour formation, it can potentiate intestinal tumour formation in mice exposed to AOM/DSS.

Talamini et al. (2019)

Test material: E 171 (35% nano), anatase, 201 nm in suspension (NTA).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure examined: quantitative analysis in tissues with methodology reliable with some limitations.

Eight-week-old male NFR mice were housed in pathogen-free animal rooms. Mice were randomly divided in to two groups (22 animals/group). One group was orally treated with 5 mg E 171/kg bw, freshly dispersed in water, and control animals received water. Mice were treated 3 days/week for 3 weeks. According to this treatment schedule, mice received an average daily dose of approximately 2 mg/kg bw per day. On day 21, three days after the last dose, mice were sacrificed and lung, liver, stomach, spleen, kidney, brain, testes and whole intestine were excised. At autopsy, the organs were sampled for each mouse, fixed in formalin, processed, embedded in paraffin and sections cut and stained with H&E or further processed for immunohistochemical analyses. Immunohistochemical staining for tissue monocytes/macrophages was performed utilising an anti-F4/80 specific antibody. Necroinflammatory foci in the liver were manually identified by three different operators blinded to treatment allocation and quantified using ImageScope software. Cytokine mRNA expression was evaluated by quantitative real-time PCR in stomach, intestine and liver. The levels of IL-1b, TNF-α and IL-6 in plasma samples were analysed with the MESO QuickPlex SQ 120, while SDF-1 (stromal-cell-derived factor-1) was determined using a ELISA kit.

This study aimed to investigate whether TiO_2 is deposited in the digestive system and internal organs and whether there are any molecular and cellular alterations associated with an inflammatory response. Regarding inflammation, to assess whether TiO_2 accumulation and/or the production of superoxide in target organs may lead to histological changes, H&E and F4/80 staining were performed. Neither overt structural and morphological histological alterations nor significant recruitment of monocytes/macrophages were observed in the stomach and whole intestine in E 171-treated animals. Neither disruption of crypt structure nor atypical epithelial cell proliferation in colon was observed. No changes in spleen histology were mentioned.

In the liver, increased size of necroinflammatory foci infiltrated with F4/80 monocytes/macrophages was noted three days after the last E 171 dose (n = 4) vs. controls (n = 2), while no changes were observed in stomach or intestine of treated animals. Liver histological findings are not supported by ROS production data or cytokine levels. An increased ROS production (superoxide anion) was observed

in the stomach and in the intestine, with no changes in the liver (n = 4), of treated animals. Regarding cytokine mRNAs, as assessed by quantitative real-time PCR in stomach, whole intestine and liver, data were not striking: a very modest increase of IL-1b was observed in stomach and whole intestine of animals exposed to E 171, a decrease in TNF-α was observed in the whole intestine but in the stomach, with no changes in liver. A slight decrease in IL-10 was observed in the liver, which was not associated with an increase in pro-inflammatory cytokines (IL-1b or TNF-α). mRNA data were provided for n = 10 for IL-1b and TNF-α, n = 5 for IL-10. The levels of serum cytokines were not consistent with tissue mRNA expression. As an increase in IL-6 and SDF1, with no changes in TNF-α or IL-1b, were observed in treated animals (n = 5).

The Panel considered that analyses, which were limited to few animals (reduced power/data attrition), showed some evidence for modest inflammation which cannot be clearly identified as adverse.

Riedle et al. (2020)

Test material: E 171, anatase, 119 nm (TEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure: qualitative analysis in tissues, methodology reliable with some limitations.

C57BL/6 mice aged 6 weeks were randomly assigned to one of four diets and exposed to 0 and ≈ 1, 10 and 100 mg E 171/kg bw per day. Mice were housed conventionally and feed intake and body weight recorded biweekly. At 6, 12 and 18 weeks, six mice per group were euthanised and the GI tracts harvested. Ileal tissues containing the most distal Peyer's patch were excised and snap frozen. Peyer's patches from 18 weeks feeding were examined by confocal microscopy and SEM with EDX analysis. Remaining Peyer's patches were collected and enzymatically digested to form single-cell suspensions for analysis by flow cytometry after immunostaining for CD4, CD45R and CD8a for lymphocytes or CD11b, CD11c and CD8a for myelocytes.

The Panel considered that this study demonstrates that particles in E 171 administered via the diet are taken up by basal cells of intestinal lymphoid follicles. The Panel considered that the parameters investigated did not show an effect on the immune system or inflammation.

Pinget et al. (2019)

Test material: E 171, anatase, 30–300 nm (SEM).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure not examined.

Five to six male C67Bl/6JAusb mice were exposed by drinking water to E 171 (0, 2, 10, 50 mg/kg bw per day) for 4 weeks. Histological analysis of the gut revealed a reduction of colonic crypt length. An increase in colon macrophages and CD8 cells were observed by FACS analysis in cell suspensions prepared from colon, and increased mRNA encoding for IL-10, TNF-α and IL-6 was detected by RT-PCR in RNA extracted form colon tissue.

Rats

Bettini et al. (2017)

Two test materials: 1) E 171, anatase, 20–340 nm (118 nm) (TEM); 44.7% particles < 100 nm; 2) TiO_2 NPs (NM-105), anatase/rutile, 15–24 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 1.

Internal exposure: qualitative measurement in tissues, methodology reliable with some limitations.

Adult Wistar rats were administered by gavage 10 mg E 171/kg bw per day for one week or 100 days. In addition, a group of animals were exposed to TiO_2 NP (NM-105) 10 mg/kg bw per day for one week. Titanium was detected in the immune cells of Peyer's patches. Effects were not noted in the spleen, but in Peyer's patches dendritic cell percentage were increased, as measured by flow cytometry of cells isolated from tissue samples. This effect was transient, as it was observed 7 days

after exposure, but not at 100 days. The percentage of regulatory T cells and T-helper (Th) cells were significantly decreased at both time points in E 171 exposed animals. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T-helper (Th)-1 IFN-γ secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated *in vitro* with anti CD3/CD28 antibodies. Regarding the effects of TiO_2 NP, similar to E 171 an increase in the percentage of dendritic cells in Peyer's patches was observed with no decrease in the percentage of Tregs. Stimulation of immune cells isolated from Peyer's patches showed a decrease in T helper (Th)-1 IFN-γ secretion, while splenic Th1/Th17 inflammatory responses sharply increased, as measured in cells taken from exposed rats, stimulated *in vitro* with anti CD3/CD28 antibodies. Regarding intestinal mucosal inflammation, E 171 for one week did not initiate intestinal inflammation, but a 100-day E 171 treatment promoted colon microinflammation evidenced by significantly increased IL-1β, IL-8 and TNF-α expression in the colon. In the same samples, increased IL-10 was also observed. Data on the effects of TiO_2 NP on intestinal mucosa were not presented. In this study, changes (aberrant crypts) were examined in the colon after staining with methylene blue. The authors did not explicitly define an aberrant crypt foci (ACF) but the Panel presumed it was 1-or more aberrant crypts/ACF. The authors defined a 'large ACF' as consisting of more than three aberrant crypts per ACF. In this study, changes (aberrant crypts) in the colon were examined. The authors provided morphological data demonstrating aberrant crypts after staining with methylene blue. The authors did not explicitly define an aberrant crypt foci (ACF) but the Panel presumed it was 1-or more aberrant crypts/ACF. The authors defined a 'large ACF' as consisting of more than three aberrant crypts per ACF.

In the first experiment, in 12 male rats pretreated with a single injection (180 mg/kg intraperitoneal in isotonic saline) of the genotoxic carcinogen dimethylhydrazine (DMH) there were on average per colon approximately 470, 190 and 30 aberrant crypts, ACF and large ACF, respectively, after 100 days. In DMH pretreated rats also subsequently (7 days later) exposed to either 0.2 or 10 mg/kg bw per day E 171 in drinking water (12 rats/group), there was a statistically significant increase per colon in number of aberrant crypts and large ACF and a statistically non-significant increase in total number of ACF in the high-dose group compared to DMN only controls. No statistically significant differences were observed between the low-dose and control groups. The incidence of ACF was not reported.

In the second experiment, male rats received either drinking water (12 controls) or 10 mg E 171/kg bw per day in drinking water (n = 11) for 100 days. No ACF were observed in the colons of controls but four rats in the treated group developed one to three ACF per colon (which in three rats consisted of 1–3 aberrant crypts/ACF with the remaining rat having 12 aberrant crypts in an ACF). The increase in the incidence of rats with ACF (4/11 vs. 0/12 in the control group) was statistically significant.

Regarding inflammation, the Panel considers that these data indicate that E 171 has pro-inflammatory potential at the systemic level, paralleled by the development of an inflammatory microenvironment in the intestinal mucosa.

The Panel considered that E 171 alone at a dose of 10 mg/kg bw per day may induce development of ACF in male rats. The Panel also noted that E 171 at a dose of 10 mg/kg bw per day increased the number of ACF initiated by a genotoxic carcinogen.

Blevins et al. (2019)

Test material: E 171, anatase, 110–115 nm (SEM), 36% particles < 100 nm.

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 3.

Internal exposure not examined.

Six-week-old male Wistar Han IGS (Crl:WI (Han)) rats were exposed to E 171 in a standard diet at a concentration of 0, 40, 400 or 5,000 mg/kg diet (equal to 1.8, 4.8, 31.4, 374 mg/kg bw per day) for 7 and (equal to 1.3, 3.5, 22.4 or 267 mg/kg bw per day[24]) 100 days. There were two different studies, one study was performed over 7 days (n = 5/group) and the other over 100 days (n = 15/group). The two studies were performed at different Institutions, with the 7-day study performed twice whereas the 100 day study was performed once. For the 7-day studies, rats were randomised into 4 groups of 5 animals and the data from the two studies were pooled. Total food and water consumption were determined at the end of each study. Body weights were determined at the start and end of the 7-day

[24] As calculated by the Panel and taking the mean of the two periods given in the paper: 1.5, 3.9, 25.5, 294 or 1.1, 3, 19, 236 mg/kg bw per day for weeks 1-10 or 11-15, respectively (groups 1–4) and 1.5, 4.1, 25.7, 300 or 1.1, 3.1, 19.2, 237 mg/kg bw per day for weeks 1–10 or 11–15, respectively (groups 5–8).

exposure period at the time of euthanasia. For the 100-day study, animals were randomised into 8 groups of 15 animals each. At the start of the study, animals in groups 5–8 were treated with a single intraperitoneal injection of a sterile dose of 180 mg/kg bw dimethylhydrazine (DMH) dihydrochloride while groups 1–4 were treated with vehicle only. Seven days after intraperitoneal injection, dietary administration of 0, 40, 400 or 5,000 mg/kg diet E 171 was started and continued for 100 days. Body weights were determined weekly beginning on day 0 of the study and just prior to euthanasia. Food consumption was determined weekly beginning with administration of the E 171 supplemented diets. Water consumption was determined during weeks 3, 8 and 13 of the study.

No significant changes in food intakes or body weights or liver and spleen weights were found and no mortality was observed. A trend towards increased food consumption in rats of the high E 171 group was observed. According to the authors, dietary E 171 produced no general signs of overt toxicity at the highest dose tested, over 100 days.

The objectives of the study were to evaluate the acute (7 days) and subchronic (100 days) effects of dietary E 171 exposure on the immune system of the GI tract and periphery as well as to evaluate effects of the subchronic exposure either alone or after pre-administration of a known intestinal genotoxic carcinogen, DMH. Concerning the latter an examination of colon for presence of aberrant crypt foci (ACF) and of aberrant crypt (ABC) was included. Concerning the effects on the immune system, the following parameters were investigated: phenotyping of immune cells (i.e. $CD103^+$ DC, total and activated T helper cells, total and activated Treg cells) and inflammatory cytokines [(IL-1α, IL-1β, IL-6, interferon γ (IFNγ), IL-12p70, IL-17A, IL-18, IL-33. CCL2/MCP-1, CXCL1/KC (IL-8), GM-CSF and tumour necrosis factor α (TNF-α)).

Following the 7- and 100-day feeding periods, rats were euthanised and measurements of inflammatory cytokines (using the LEGENDplex rat inflammation Panel) and phenotyping of immune cells (by flow cytometry) in the periphery and GI tract were performed. Peyer's patches, peripheral blood mononuclear cells (PBMC) and spleen cells were analysed for inflammatory and regulatory T-cell responses directly *ex vivo* or after *in vitro* stimulation with anti-ratCD3 (5 μg/ml) and anti-rat CD28 (5 μg/ml) for 4 days. Histopathology, ACF, ABC and goblet cell evaluations were performed on rats in the 100-day study. All tissues were collected from well-defined areas, and measurements, procedures and evaluations were performed in a standardised and blinded manner.

$CD103^+$ dendritic cells (DC) were evaluated in the gut, Peyer's patches, spleen and in peripheral blood over time period. No change in the percentage of $CD103^+$ DC in peripheral blood, spleen or Peyer's patches due to acute or subchronic dietary E 171 consumption alone was observed.

The total percentage of $CD4^+$ T helper cells, the percentage of T helper cells expressing CD25, an indicator of T helper activation, and the percentage of Treg cells ($CD4^+FoxP3^+$) and activated Treg ($CD4^+CD25^+FoxP3^+$), critical mediators of local and systemic inflammation, which could lead to a low level inflammatory response in the absence of increased inflammatory cells, were quantified in peripheral blood, spleen and Peyer's patches. Dietary E 171 exposure did not change the frequency of $CD4^+$ T helper cells systemically or in intestinal Peyer's patches. In addition, there was no detectable impact on the percentage of activated $CD4^+$ T helper cells or on the percentage of Treg cells either peripherally or locally in the Peyer's patches of treated rats fed for 7 or 100 days. Collectively, these results suggest that E 171 consumption does not alter T-cell-mediated mechanisms of immune control, either promoting inflammatory $CD4^+$T helper cell activation or in reducing the percentage of anti-inflammatory Treg cells.

Regarding the effects on cytokines, data presented suggest that dietary E 171 does not induce inflammation peripherally or in the GI tract at both time points. In addition, studies were conducted to explore the possibility that E 171 might alter the effector cytokine profile of T helper cells in lymphoid tissue or circulation, which may not be manifest without T cell-specific stimuli. Lymphocytes were isolated from peripheral blood, spleen and Peyer's patches and activated *ex vivo* with anti-CD3/anti-CD28 for 4 days to induce T helper cell cytokine production. No effects of E 171 exposure on any of the induced cytokines produced from *ex vivo* stimulated T helper cells were observed.

In the 100-day study, all animals were treated with E 171, some groups were initiated with 180 mg/kg bw DMH before the start of the dietary exposure to E 171 and an additional control initiated with DMH was also included. The same parameters as described above were evaluated, with some differences observed. A modest increase in the relative spleen weight in 22.4 mg E 171/kg bw per day + DMH compared to not initiated animals, an increase in IL-17A in colon (22.4 mg E 171/kg bw per day + DMH) and IL-12p70 in plasma (3.5 mg E 171/kg bw per day + DMH), with no dose-related effects, were observed. There were no changes in spleen cellularity across any of the treated groups. No changes were observed in the percentage of $CD103^+$ DC, $CD4^+$ T helper cells or total or activated

Treg in peripheral blood, spleen or Peyer's patches in animals exposed to E 171 + DMH compared to animals treated with only DMH.

According to the authors, there were no treatment related histopathological changes in the duodenum, jejunum, ileum, spleen, liver, lung and testes in animals exposed only to E 171. Rats that were initiated with DMH only and those which received 171 in the diet after the initiation displayed several histopathological abnormalities. There were two invasive adenocarcinomas in one animal in the 1.3 mg E 171/kg bw per day + DMH group, and single adenomas in one animal in the 3.5 mg E 171/kg bw per day E 171 + DMH group and in one animal in the 22.4 mg E 171/kg bw per day + DMH group. There were no other histopathological changes in the large intestines of the other animals treated with DMH. One rat in the 1.3 mg E 171/kg bw per day + DMH group and one rat in the 22.4 mg E 171/kg bw per day + DMH group had subpleural lymphocytes in the lung, but without any evidence of acute inflammatory changes or hyperplasia.

Regarding colonic ACF and ABC, according to the authors, a technical issue was encountered in that much of the epithelial surface of the sampled colon (proximal, middle and distal) was obscured when observed by light microscopy. The authors' reported that they were unable to examine the entire surface of the colon samples. The results for the areas of epithelium that were examined indicated an increase in ACF/cm^2 and ABC/cm^2 in groups initiated with DMH compared to the groups that were not initiated with DMH. E 171 treatment administered after DMH did not result in statistically significant increases in ACF or ABC. No change in the number of ACF and ABC were observed due to E 171 exposure alone. The Panel noted a considerable variability in the results, which may mask possible effects. Furthermore, the Panel noted that the examination for presence of ACF and ABC was not performed on the whole colon but was limited to three 2 cm long samples (one from the proximal, mid-portion and the distal parts). Dietary E 171, with or without treatment with DMH, had no effect on the length of the colonic glands examined or the number of goblet cells/unit.

Overall, the Panel considered that this study indicates that acute and subchronic dietary intake of E 171 resulted in no significant effects on either peripheral or GI tract immune homeostasis as evidenced by immune cell phenotyping or inflammatory cytokine analysis.

Limitations in the pathological examination for ABC and ACF (sampled colon area limited; delayed fixation) preclude a conclusion on potential for ABC and ACF formation.

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Test material: E 171, anatase, 150 nm (DLS).

Scoring for nanoscale considerations (dispersion and/or confirmation of internal exposure), assigned according to Appendix E, was 2.

Internal exposure: quantitative analysis in tissues; methodology not reliable.

A 90-day study was conducted according OECD TG 408. E 171 (10 rats/sex/dose) was administered daily by gavage for 90 days to Sprague–Dawley rats (0, 10, 100 and 1,000 mg/kg bw per day). Clinical signs of all rats were observed daily during the study period.

Regarding immunological parameters: blood levels of immunoglobulin (Ig) A, IgE, IgG, IgM and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured. In addition, total and differential white blood cell (WBC) counts and immune organ weights and histology were also evaluated. There were no changes in the weight of immune organs or their histology. The proportion of lymphocytes slightly decreased (by 9%) in male but not female rats administered the highest E 171 dose, without an apparent dose response relationship. According to the study authors, the level of GM-CSF was reduced by 41% in females only at the highest dose of 1,000 mg/kg bw per day. In males there was also a decrement in GM-CSF level (approximately by 30%), that was slightly less pronounced than in females, and due to a higher variability in the controls, did not gain statistical significance. A reduction in the levels of IgM was observed in both sexes at the highest dose tested, i.e. by 12% in females and by 9% in males, but there were no clear dose response relationships. Transcriptomics also showed that immune response-related microRNAs were most strongly affected by E 171 exposure, which may support the effect observed at the high dose.

The Panel noted the lack of a dose response, the magnitude of the effect was small that did not allow a firm conclusion given the natural variability in the parameters measured.