

Beyond the white

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Chapter 7

Summary and general discussion



Colorectal cancer (CRC) is the second most prevalent cancer in women with 614,000 cases (9.2% of the total number of cancer cases) and the third in men with 746,000 cases (10.0% of the total number of cancer cases) in 2012 worldwide (1). Strong evidence was found between dietary factors and cancer risks (2). The consumption of milk and whole grains products may have a protective role against CRC (2) whereas consumption of red and processed meat may increase cancer risks by stimulating the endogenous formation of carcinogenic N-nitroso compounds (NOCs) and the presence of pyrolysis products like polycyclic aromatic hydrocarbons (PAHs) heterocyclic amines (3). In recent years, concerns about the potential adverse effects of titanium dioxide (TiO₂) as food additive are rising in scientific literature. Two different crystal forms are authorised in food products: anatase and rutile. Due to lower production costs, anatase is the form the most commonly used in food and mostly studied. The anatase TiO₂ food additive is designated E171. The food additive E171 comprises between 25-40% of NPs (<100 nm) and 60-75% of microparticles (MPs) (>100 nm) (4-7).

Exposure to TiO₂ by ingestion of food and products containing TiO₂ occurs on a daily basis and several studies showed that people of all ages from infant to elderly are exposed (7-10). Exposure to TiO₂ happens as a consequence of the ingestion of many food products like dairy products (milk, cheese, and ice cream), milk replacements (powdered milk), sweets (M&M's[®], Mentos[®], chewing-gums, and cookies), drinks (soft drinks, sport drinks, and syrups), dressings and sauces (salad dressings), and food supplements (multivitamin pills) (7-11).

In vivo studies after ingestion of E171 show an increased tumour formation and the growth of aberrant crypt foci in a chemically induced CRC mouse and rat models respectively (12,13). Furthermore, in normal BALB/c mice exposed to 5 mg/kg_{bw}/day of E171, dysplastic changes in the colonic epithelium and a decrease of goblet cells were observed (13). In addition, after ingestion of 10 mg/kg_{bw}/day of E171 in rats for 7 days a Th1/Th17 immune deviation as well as an impairment of the intestinal homeostasis was observed (12). In the same study, the identical effects on the immune response and intestinal homeostasis were observed after ingestion of 10 mg/kg_{bw}/day of anatase TiO₂ nanoparticles (NPs). The NPs have a smaller size (<100 nm) and a higher surface area, therefore, these particles are assumed to have the most adverse effects. Consequently, a majority of the studies on adverse effects of TiO₂ have been performed with the anatase NPs fraction. After *in vivo* and *in vitro* exposure to TiO₂ NPs, observations of effects towards a facilitation of the development of cancer were supported by findings of an increased oxidative stress (4,14), an induction of DNA damage (4,15-17), and an

impairment of the immune system (18,19). In addition, an *in vivo* study on the adverse effects of rutile NPs showed an inflammatory response via cytokine production or NLRP3 inflammasome in the gut (20).

Emerging evidence of adverse effects of TiO₂ have raised concerns about the safe use of TiO₂. Therefore, the International Agency for Research in Cancer (IARC) evaluated TiO₂ in 2010 based on new findings (21). Human epidemiological studies on inhalation were assessed and the agency decided that the data available was inadequate to draw a conclusion whether or not TiO₂ causes cancer in humans. Animal studies on inhalation were also evaluated and the IARC concluded that there was convincing evidence of an increase of lung tumours. Other routes of administration (ingestion and dermal contact) did not show an increase incidence of tumours. Subsequent to the results of these studies, the IARC classified TiO₂ as possible carcinogen to humans (Group 2B). The modes of action underlying these adverse effects like kinetics, *in vivo* and *in vitro* genotoxicity, cytotoxicity, presence of inflammation, and penetration of TiO₂ through the skin have also been taken into consideration for the evaluation. The evidence was not strong enough to warrant classification other than Group 2B.

Traditionally toxicity testing of products and compounds was only based on only animal testing with a specific end-point. Currently, *in silico* methods are added to the animal testing to evaluate mechanistic changes in biological processes using biopsies, cells, and organs. Advances in bioinformatics, epigenetics, toxicogenomics, systems biology, and computational toxicology are transforming the toxicological assessment of compounds. The combination of these scientific advances with animal testing with a specific end-point are expected to generate more robust data on the potential risks to humans and offering the prospect of improved risk-based regulatory decision (22,23). Therefore, elucidating the molecular mechanisms behind the adverse effects of E171 is becoming an important step for food safety evaluation. Mechanistic toxicology or toxicogenomics, through the identification and interpretation of significant gene expression changes, may play an important role in linking cellular response to toxicological endpoints (22,23). Within the realm of toxicogenomics, a technique available is the high-throughput analysis of biological systems: whole-genome mRNA microarrays used for transcriptomics analysis.

We aimed in this thesis to 1) better understand the potential risks of the TiO₂ food additive E171 by studying the molecular mechanisms of facilitation of CRC as well as the histopathological changes observed after exposure to E171 *in vivo* and 2) to assess the relative contribution of the different fractions of E171 to the adverse effects *in vitro*. For these purposes, we applied transcriptomics analyses in combination with markers of cytotoxicity, genotoxicity and oxidative stress.

I- Potential risks of ingestion of TiO₂

I-1- *In vivo* studies

In order to answer the first research question of this thesis, gaining insight in the mechanisms of facilitation of CRC as well as the pathological changes observed after exposure to E171, 3 *in vivo* studies were performed. All *in vivo* experiments were performed with the food additive E171. To optimise the evaluation of the mechanisms behind the adverse effects of E171, common conditions of exposure *in vivo* were chosen in this thesis i.e. sample preparation, type of ingestion, time points of sampling, and types of samples for microarray analysis. The time points chosen were based on the long term exposure performed by Urrutia-Ortega et al. (13). A significant increased number of tumours in the distal colon was observed in the chemically induced CRC mouse model with azoxymethane (AOM) and dextran sodium sulphate (DSS) additionally exposed to 5 mg/kg_{bw}/day of E171 for 10 weeks. In the same study, in the mice solely exposed to E171, dysplastic changes in colonic epithelium and a decrease of goblet cells were observed. The tumours arise after 3 weeks of exposure to E171 in combination with AOM/DSS, therefore, 4 time points were chosen before tumours arise at 2, 7, 14, and 21 days. Transcriptomics changes were determined in the distal colon of mice at each time point, where the tumours were previously found. All *in vivo* experiments were performed in these conditions with the same concentration of E171 given by intragastric administration: 5 mg/kg_{bw}/day. Only in the mouse model of **Chapter 4**, 2 additional concentrations 1 and 2 mg/kg_{bw}/day were used. Three mouse models were chosen: a normal BALB/c model (**Chapter 2**), a chemically induced CRC model with AOM/DSS (**Chapter 3**), and a transgenic Cre-LoxP with Car-1 promoter model (**Chapter 4**).

In the study described in **Chapter 2**, we aimed to unravel the mechanisms underlying the pathological changes observed by Urrutia-Ortega et al. after sole exposure to E171 (13). Normal BALB/c mice were exposed to E171 and gene expression changes were observed as well as histopathology in the distal colons. Histopathological analysis showed alteration and disruption in the normal structure of crypts inducing a hyperplastic

epithelium. At the mRNA level, significant gene expression changes were observed in oxidative stress, the immune system, the olfactory/GPCR receptor family and of cancer related genes.

A majority of the biological effects observed in the normal BALB/c mice (**Chapter 2**) were also observed in the experiment described in **Chapter 3**, a study aiming to understand the mechanisms behind the development of tumours observed by Urrutia-Ortega et al. (13) in a chemically induced CRC model after E171 ingestion. Similar affected biological processes in normal BALB/c (**Chapter 2**) and in the CRC mouse models (**Chapter 3**) were observed including the downregulation of genes involved in the immune system, suggesting an impairment of this system. Also changes in the oxidative stress and olfactory/GPCR receptor family were found in both normal and CRC models. In addition, in the experiment in which mice were stimulated to form tumours by AOM/DSS described in **Chapter 3**, significant changes in the mRNA levels were identified for genes involved in biotransformation of xenobiotics which can form reactive intermediates resulting in toxicological effects.

In the study described in **Chapter 4** which was aiming to confirm the results observed in normal BALB/c (**Chapter 2**) and in the CRC mouse models (**Chapter 3**) in a transgenic mouse model that spontaneously develops colorectal tumours, similar biological processes affected after E171 exposure were observed. In this model the Cre-LoxP with Car-1 promoter leads to spontaneous colon cancer formation in 26% of the animals. An increase of the number of tumours per mice and the number of mice with tumours was observed after exposure to 5 mg/kg_{bw}/day. Furthermore, after exposure to 1 mg/kg_{bw}/day of E171, gene expression changes were observed genes involved in cell cycle, signalling, cancer, DNA repair, gene expression (transcription), the immune system, and inflammation pathways.

Gene expression changes related to signal transduction, immune system, oxidative stress, and cancer-related genes reflect early biological responses induced by E171 which precede tumour formation in an AOM/DSS mouse model (13) and are in line with the pathological changes observed in the colon of rats and mice after E171 exposure (12,13). In addition, modulation of genes involved in the immune system is in line with a recent study which described an impairment of the intestinal immune homeostasis after oral exposure to E171 for one week (12).

It should be mentioned that transcriptomics analysis is still limited to the current knowledge of the functionality of genes and their involvement in pathways. Indeed, in this thesis, we report changes in genes that have thus far not been classified in known pathways but may induce functional changes by interacting with other genes involved in known biological pathways. Moreover, accumulation of E171 in other organs than the gastrointestinal tract can induce diverse effects like reduced fertility (24), decreased glucose levels in blood (25,26), and formation of ROS and DNA damage in different organs (15-17).

1-2- in vitro studies

In order to address the second aim of this thesis, the relative contribution of the different fractions of E171 to the adverse effects, 2 *in vitro* studies were performed. Effects of the MPs and NPs fraction of E171 have been assessed *in vitro* using Caco-2 cells in **Chapter 5 and 6**. From the biological effects observed, the relative contribution of the different sizes *in vivo* may be inferred. First, in **Chapter 5**, the cytotoxicity of the E171, NPs, and MPs was studied with a Trypan blue test, the genotoxicity with comet assay and micronucleus test, and the capacity to induce oxidative stress by electron spin spectroscopy. Results showed that E171 was cytotoxic to Caco-2 cells from a concentration of 14.3 $\mu\text{g}/\text{cm}^2$ whereas NPs and MPs was cytotoxic at a concentration of 143 $\mu\text{g}/\text{cm}^2$ suggesting that the combination of NPs and MPs in E171 was more cytotoxic than the NPs and MPs separately. In addition, E171 and NPs induced reactive oxygen species (ROS) formation in a cell-free environment whereas MPs induced ROS in presence of Caco-2 cells after 1h exposure. Chromosome damage was shown to be induced by E171, as tested with the micronucleus assay. In addition, single-strand DNA damage was observed for all 3 different TiO_2 suggesting that both NPs and MPs fractions contribute to the adverse effects of E171. In line with our results, MPs were also found to induce oxidative stress in RAW 264 cells after 4h of exposure (27). More recently, the capacity of E171 to induce ROS formation and DNA damage was confirmed in Caco-2 and Caco-2/HT29-MTX cells (4). In addition, the capacity of TiO_2 NPs to induce oxidative stress is also reported in several cell lines like mouse fibroblast, mouse peritoneal macrophages (RAW 264), goldfish skin cells (GFSk-S1), human bronchial epithelial (BEAS-2B) cells, human bronchial fibroblasts (IMR 90), human foetal osteoblast cell, human amnion epithelial (WISH), glial, brain microglia, and human epidermal cells (16,27-39). In addition, Charles et al. evaluated a number of studies that had an appropriate level of confidence suitable for regulatory context i.e. studies with adequate characterization of TiO_2 , adequate description of the dispersion and genotoxicity protocols, evidence of cytotoxicity, inclusion of positive and negative results, and use of replicates or independent experiments (27).

The 36 studies assessed showed that 58% of the comet assays reported positive results as well as 56% of the micronucleus assay after exposure to anatase TiO₂ NPs. These results show that potential *in vivo* genotoxic effects might be underestimated, therefore, further evaluation needs to be performed by *in vivo* studies in combination with genotoxic tests on different organs.

Because of its small size and higher surface area, the NPs fraction is suspected to be the most active fraction. Therefore, after a time course exposure with TiO₂ NPs *in vitro* to determine the best exposure time, exposure to E171 and MPs was performed in **Chapter 6**. At the optimal time point, 24h, gene expression changes in Caco-2 cells exposed to NPs, MPs and E171 were established. Overall effects after E171, NPs, or MPs exposure *in vitro* were gene expression changes related to signalling, inflammation, immune system, and cancer. The results showed that E171 induces more changes at the mRNA level than the MPs or NPs fractions. In addition, in **Chapter 6**, effects of NPs and E171 exposure suggested that some similar biological processes were affected: TLR cascade, MHC class I and II presentation, late cornified envelope, potassium channels and cell cycle. In addition, after exposure to MPs and E171, identical affected biological processes were observed: Hedgehog family, α -defensins, cadherin and cholinergic receptors. Most studies on TiO₂ are based on the NPs fraction of E171, but as shown in the *in vitro* studies on the relative contribution of the NPs and MPs fraction to the effects of E171 (**Chapter 5 and 6**), the MPs fraction can also induce adverse effects. Even if the NPs induced more pronounced effect at the transcriptome level compared to the MPs, both fractions included in E171 should be assessed. The gene expression changes related to signalling, inflammation, immune system, and cancer confirm previous findings described in normal BALB/c mice (**Chapter 2**), CRC mouse model (**Chapter 3**), and transgenic mouse model (**Chapter 4**). Furthermore, the immune response genes affected after exposure to TiO₂ in Caco-2 cells shows that this biological process might not only be induced in combination with other organs as seen *in vivo* in BALB/c mice, CRC mouse model, and transgenic mouse model but also as a consequence of direct exposure of intestinal epithelium cells.

I-3- Additional biological processes

In the studies described in this thesis, we have also demonstrated that the adverse effects induced by E171 are very diverse. In addition to processes mentioned above in normal BALB/c mice (**Chapter 2**), in CRC mouse model (**Chapter 3**), and in transgenic mouse model (**Chapter 4**), we also describe gene expression changes in Cyp450 genes, metabolism, insulin metabolism, transport of molecules, and bone development. In the colon of normal BALB/c mice exposed to E171, mRNA was described to be modulated in pathways indicating calcium mobilisation, cell cycle, and metabolism of RNA. In the colon of CRC mice exposed to E171, transcriptome changes in bile metabolism, digestive system, haemostasis, metabolism of xenobiotics, extracellular matrix organisation, muscle contraction, and translocation were also observed. In the colon of the transgenic mice exposed to E171, gene expression changes identified related to circadian clock, endocrine system, cell cycle, haemostasis, metabolism of xenobiotics, and extracellular matrix organisation. Furthermore, in the *in vitro* experiment with Caco-2 cells exposed to E171, NPs, or MPs (**Chapter 6**) additional biological processes were also observed. Gene expression changes in neuronal system and transport of small molecules were also reported after NPs exposure. After MPs exposure, additional affected biological processes were in developmental biology. After E171 exposure, modulation of mRNA levels in metabolism of proteins, developmental biology, transport of small molecules and haemostasis were observed.

Overall, the diversity of molecular mechanisms influenced by E171 in colon shows that E171 is not inert and the adverse effects may not only contribute to cancer development in colon but may also aggravate inflammatory bowel diseases.

II- Relevance to the human situation

The concentrations used during the *in vivo* and *in vitro* studies described in this thesis and aimed to represent the actual human exposure. Weir et al. showed that adults (>10 years old) are exposed to 0.2-0.7 mg/kg_{bw}/day in the US and 1 mg/kg_{bw}/day in the UK (7). A Dutch study estimated an average exposure of 0.17 mg/kg_{bw}/day of TiO₂ (11). A higher exposure has been estimated by the EFSA in their re-evaluation of TiO₂: 2.4 mg/kg_{bw}/day (8). The quantity of TiO₂ ingested in children is higher than in adults because of the higher quantities of sweets and cookies eaten by the children as well as ingestion of toothpaste. The estimated ingestion of TiO₂ for children (<10 years old) in US is 1-2 mg TiO₂/kg_{bw}/day and in UK 2-3 mg TiO₂/kg_{bw}/day (7). In addition, the Dutch estimation was of 0.67 mg TiO₂/kg_{bw}/day between 2-6 years old (11) and the EFSA estimated the ingestion at 5.5 mg/kg_{bw}/day for children between 3-9 years old (8).

The highest concentration used in this thesis was 5 mg/kg_{bw}/day which is in the same range as the human exposure, closer to the children exposure. In the study described in **Chapter 4**, 3 different concentrations of E171 were studied and the strongest effects were observed with the lowest concentration (1 mg/kg_{bw}/day) compared to 2 and 5 mg/kg_{bw}/day. The lowest concentration used in this **Chapter 4** is the closest to the US, UK, and Dutch estimation for adults and lower than the one from the EFSA.

As for the relevance of the *in vitro* concentrations to the human situation, a conservative estimate was performed in **Chapter 5**. In this scenario, a 70 kg adult was assumed to eat 1 mg/kg_{bw}/day. In the 250 g faeces excreted, the concentration of E171 would be 0.28 mg/g of faeces. As faeces contain 75% of water and if we consider the density of faeces to be similar as water, the concentration of E171 would be 0.28 mg/mL of faeces. By assuming that 1% of this is biologically available, 0.0028 mg/mL would be potentially the concentration in contact with the gut cells. The concentration used in **Chapter 5 and 6** were 0.01 and 0.001 mg/mL. These concentrations are in the same order of magnitude as the estimated concentration in humans and were first tested to be non-cytotoxic in Caco-2 cells (**Chapter 5**).

The estimated daily exposure and the conservative scenario do not include exposure to TiO₂ via the ingestion of pharmaceutical pills. Depending on the properties of the pill, they could dissolve in very different parts of the gastrointestinal track. One can therefore assume that the intestinal epithelium can be exposed to higher concentration of TiO₂ depending on the pill ingested in addition to E171.

III- Conclusions and future perspectives

The results of this thesis show the complexity of the response to the exposure to E171. Whole-genome gene expression analysis on colonic tissue as well as *in vitro* testing indicated mechanisms by which E171 may enhance tumour formation (13). Transcriptome changes have demonstrated the presence of oxidative stress, DNA damage, impairment of the immune system, and activation of cancer-related genes as a consequence of exposure to E171, which may collectively represent the molecular mode of action that explains the stimulation of colorectal tumour formation. Moreover, the information provided by this thesis indicates that the relative contribution of each fraction to these effects is different. The NPs fraction by its small size and higher surface area seems to induce more adverse effects than the MPs. Yet, the fact that MPs have an effect on ROS, DNA damage, and gene expression changes implies that potential health risks cannot be eliminated by increasing the proportion of MPs in E171.

The results of this thesis generated more insight of the potential adverse effects following oral exposure to E171. However, for a full risk assessment, additional experiments should be performed. It is important to establish causality between the gene expression changes and processes leading to CRC development, which requires the *in vivo* investigation of the functionality of the immune system, inflammation, oxidative stress, and DNA damage. Such studies may be also done according to the Organisation for Economic Co-operation and Development (OECD) guidelines for regulatory purposes. Repeated dose 28-day and 90-day oral toxicity studies in rodents should be performed. In order to have more insight in the effects of exposure on the immune system, an extended one generation reproductive toxicity study should be performed, which includes exposure during all life stages of the animals, and includes a specific cohort of animals tested for developmental immunotoxicity.

Additional *in vitro* and *in vivo* experiments would help to extrapolate the enhancement of tumour growth observed in the animal studies to real life exposures, i.e. consumers ingesting E171. Additional *in vitro* models like co-cultures of different cells and cell lines (e.g. Caco-2 and macrophages) or 3D models with normal human colon organoids and spheroids can serve for this purpose. The consistency found between different *in vitro* models with the combination of several human cell lines allows a closer understanding of the human situation and therefore improving the extrapolation. Eventually, a randomized controlled intervention study in humans on E171 ingestion should be performed, in which gene expression profiles in the colon would be analysed. Such information would

substantiate the relevance of the *in vitro* findings and animal data, and provide the definitive proof of adverse effects of ingestion in the population. The totality of the evidence, including dose response relationships of the endpoint and intermediate measures in animals, *in vitro* studies, and studies on early effects in humans will provide a more solid base for estimating the risk of colon tumour enhancement after intake of E171.

In order to further advance our insight in mechanisms underlying the effects, novel technologies could be applied. All findings described in this thesis are based on microarray analysis, however, the use of technologies such as sequencing may be instrumental for this purpose. This technique allows more in depth as well as quantitative analyses of effects of TiO₂ on the expression of mRNA, transcript isoforms, non-coding RNA, and circular RNA. In addition, modulation of miRNAs expression can lead to upregulation of miRNA involved in cancer or downregulation of tumour suppressors which would enhance cancer development (39). Such regulators of cancer-related processes can be identified by miRNA-sequencing in blood and colon tissue of animals and humans after exposure to E171 and would contribute to the understanding of mechanisms behind the enhancement of CRC by E171. Furthermore, time-series analysis would help to identify gene expression patterns over time and dose which allows for evaluation of the mechanisms underlying the effects following oral exposure to E171. Applying Dose-Time Network Identification (DTNI) tool for data analysis would provide more in depth information on inferring molecular key events and may detect novel interactions which can be studied experimentally by silencing these genes (40).

Based on the new information presented in this thesis, we conclude that the classification of E171 as free from toxic effects on the account of its insolubility and inertness is no longer valid. Furthermore, the results of this thesis indicate the presence of inflammation that was found in animal models after E171 ingestion could aggravate inflammatory bowel diseases and potential adverse effects towards enhancement of colorectal cancer. Therefore, we recommend that the experiments described here above, with an emphasis on actual testing in humans, should be performed for a further evaluation of E171 on its potential adverse effects on the enhancement of cancer, dysregulation of the immune system, and inflammation. These new data would provide insight on the effects on humans for a full risk assessment which can lead to a modification of the use of E171 in food products. These modifications may be a reduction of the quantity of NPs, establishing a maximum level of use in food products, a stricter limitation in the types of products it can be used in, or a banishment of the product itself.

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