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In Vitro Toxicity Assessment of Metal Nanoparticles in Intestinal and Placental Cells

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Abstract

Introduction: Metal nanoparticles (M-NP) are among the most widely used nanomaterials (NM) in several areas such as industry, environment, agriculture, and biomedicine. Thus, human exposure to these nanosized materials is increasing, which raises serious concerns regarding their environmental and human safety. Biological barriers are important lines of defence to xenobiotics, thus expected targets for M-NP. The tiny size of NM allows them to pass more easily across biological barriers, so they can be potentially toxic.

Aims: The present study aimed at evaluating the main mechanisms of *in vitro* toxicity of different M-NP in two cell models of biological barriers: human intestinal (Caco-2) and trophoblastic (BeWo clone b30) epithelial cells.

Methods: Cells were exposed for 24 h to varied concentrations (0.8 - 48 μg/cm^2) of M-NP of different chemical composition (Au, Ag and TiO_2), primary size (10, 30 and 60 nm), capping (citrate and PEG) and crystal structure (rutile and anatase) and toxicity assessed by determining changes in cell morphology, metabolic activity, plasma membrane integrity, generation of intracellular reactive oxygen species (ROS) and intracellular ATP levels.

Results: Our data show a size- and concentration-dependent reduction in metabolic activity and increase in extracellular LDH for AgNP. Rutile-anatase produced the same effects as anatase. Citrate-coated AuNP decrease metabolic activity and, in addition, PEG-capping effectively attenuated AuNP-induced toxicity. Additionally, only cells exposed to AgNP and PEG-AuNP exhibited significant increased levels of ROS. All tested M-NP significantly increased intracellular ATP levels compared to control cells, except for 10 nm AuNP in BeWo b30 cells.

Conclusions: The hazard ranking of the tested M-NP is similar in both cell lines with AgNP > AuNP > TiO_2NP, being the cytotoxic effects more visible at higher concentrations. The influence of the size in the cytotoxic-induced effects was more evident for AgNP than for AuNP, with the smaller NP causing more cytotoxicity, being the BeWo b30 cells more sensitive to these M-NP. Thus, our data support that the physicochemical properties of the NM are an important determinant of their cytotoxicity. Future studies will be useful to further explore the effects of M-NP in the human barriers.

Keywords: Nanotoxicology; Nanomaterials (NM); Nanoparticles (NP); *In vitro* toxicity; Biological barriers

Resumo

Introdução: As nanopartículas metálicas (M-NP) estão entre os nanomateriais (NM) mais amplamente utilizados em diversas áreas como a indústria, o meio ambiente, a agricultura e a biomedicina. Assim, a exposição humana a estes materiais nanométricos está a aumentar, o que levanta sérias preocupações em relação à sua segurança ambiental e humana. As barreiras biológicas são importantes linhas de defesa aos xenobióticos e, portanto, alvos esperados para as M-NP. O tamanho minúsculo dos NM permite que eles passem mais facilmente pelas barreiras biológicas, e serem potencialmente tóxicos.

Objetivos: O presente estudo teve como objetivo avaliar os principais mecanismos de toxicidade *in vitro* de diferentes M-NP em dois modelos celulares de barreiras biológicas: células epiteliais intestinais humanas (Caco-2) e trofoblásticas (BeWo clone b30).

Métodos: As células foram expostas durante 24 h a concentrações crescentes (0,8-48 $\mu g/cm^2$) de M-NP com diferente composição química (Au, Ag e TiO_2), tamanho primário (10, 30 e 60 nm), revestimento de superfície (citrato e PEG) e estrutura cristalina (rutilo e anatase). A toxicidade foi avaliada pela determinação de alterações na morfologia celular, atividade metabólica, integridade da membrana plasmática, geração de espécies reativas de oxigénio (ROS) e níveis intracelulares de ATP.

Resultados: Os nossos dados mostram uma redução na atividade metabólica dependente do tamanho e da concentração e um aumento da libertação de LDH nas células expostas às AgNP. As TiO_2NP rutilo-anatase produziram os mesmos efeitos que a forma anatase. AuNP revestidas com citrato diminuíram a atividade metabólica e, além disso, o revestimento com PEG atenuou efetivamente a toxicidade induzida pelas citrato-AuNP em ambos os modelos de barreira. Além disso, apenas as células expostas a AgNP e PEG-AuNP exibiram níveis aumentados significativos de ROS. Todas as M-NP testadas aumentaram significativamente os níveis de ATP intracelular em comparação com as células controlo, exceto as AuNP 10 nm nas células BeWo b30.

Conclusões: O ranking de perigosidade das M-NP testadas é semelhante em ambas as linhas celulares com AgNP > AuNP > TiO_2NP, sendo os efeitos citotóxicos mais visíveis em concentrações mais elevadas. A influência do tamanho na citotoxicidade foi mais evidente para as AgNP do que para as AuNP, com as NP menores a causarem mais citotoxicidade, sendo as células BeWo b30 mais sensíveis a estas M-NP. Assim, os nossos dados suportam que as propriedades físico-químicas dos NM são um importante determinante da sua citotoxicidade. Estudos futuros serão úteis para explorar ainda mais os efeitos das M-NP nas barreiras humanas.

Palavras-chave: Nanotoxicologia; Nanomateriais; Nanopartículas; Toxicidade *in vitro*; Barreiras biológicas

In the course of this work, the following review article was published:

How can exposure to engineered nanomaterials influence our epigenetic code? A review of the mechanisms and molecular targets**.** Moreira, L., Costa, C., **Pires, J.**, Teixeira, J. P., Fraga, S. (2021). *Mutation Research: Reviews in Mutation* Research, 788, 108385. https://doi.org/10.1016/j.mrrev.2021.108385

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***In vitro* Toxicity Assessment of Metal Nanoparticles in Intestinal and Placental Cells**

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Comparative assessment of the acute toxicity of commonly used metal nanoparticles in two *in vitro* models of human barriers

Pires, J., Moreira, L., Teixeira, J. P. and Fraga, S.

EuroNanoForum 2021 (online conference), 5th – 6th May 2021, Braga, Portugal. *Poster Presentation*

***In Vitro* Toxicity of Metal Nanoparticles in Two Human Barrier Models: Role of the Physicochemical Features**

Pires, J., Moreira, L., Teixeira, J. P. and Fraga, S.

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***In Vitro* Acute Toxicity of Metal-Based Nanoparticles in Human Trophoblast BeWo b30 Cells**

Pires, J., Moreira, L., Teixeira, J. P. and Fraga, S.

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List of Abbreviations and Acronyms

ABBREVIATION	DESCRIPTION
AgNP	Silver nanoparticle
ATP	Adenosine triphosphate
AuNP	Gold nanoparticle
AuNP_10_PEG	PEGylated gold nanoparticle
Carboxy-DCFH-DA	Carboxy-2',7'-dichlorodihydrofluorescein diacetate
CNP	Carbon nanoparticles
DCF	2',7'-dichlorofluorescein
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetraacetic acid
EtOH	Ethanol
FBS	Fetal Bovine Serum
GIT	Gastrointestinal tract
Nano_GO	Nano-Graphene Oxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LDH	Lactate dehydrogenase
mM	Millimolar
M-NP	Metal Nanoparticle
NaF	Sodium Fluoride
NP	Nanoparticle
rcf	Relative centrifugal force
ROS	Reactive Oxygen Species
SD	Standard Deviation
SiO_2NP	Silica nanoparticle
TiO_2NP	Titanium dioxide nanoparticle

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1. Introduction

1.1. The nanotechnology revolution and nanomaterials (NM)

Since the beginning of the 1990s, one of the main technologies used nowadays, the nanotechnology, has experienced an enormous growth giving rise to various types of nanomaterials (NM) (Bayda *et al.*, 2019; Gupta and Xie, 2018). Nanotechnology involves the creation, manipulation, characterization, production and application of particles, engineering materials, products and devices that are unique or enhanced at the nanoscale (Bayda *et al.*, 2019; Gupta and Xie, 2018; Maynard *et al.*, 2006). Numerous definitions of NM have already been proposed by governmental, industrial and standardization organizations (Bleeker *et al.*, 2013; Boverhof *et al.*, 2015). According to the European Commission (EU), a NM is defined as a "natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm" (Commission, 2011). Therefore, nanotechnology is a very promising field, which provides great potential for the future, considering the technological advances that can result from its use in a wide range of industrial areas.

The increasing use of NM for commercial purposes is essentially due to their remarkable properties that arise from their high surface area to volume ratio compared to their bulk counterparts (Arora *et al.*, 2012). This aspect endows NM of physical, chemical, biological, magnetic, mechanical, catalytic, thermal, optical, electrical, and luminescent properties that are "new and unique". Many materials such as nanowires, nanoparticles (NP), nanofibers, nanotubes, composite materials, and nanostructured surfaces are available.

The growing interest in research on NM is represented in **Figure 1**, showing the number of annual publications in the PubMed database (National Library of Medicine) between 1990 and 2021 using as subject headings "nanomaterials" and "nanoparticles" and using the AND operator.

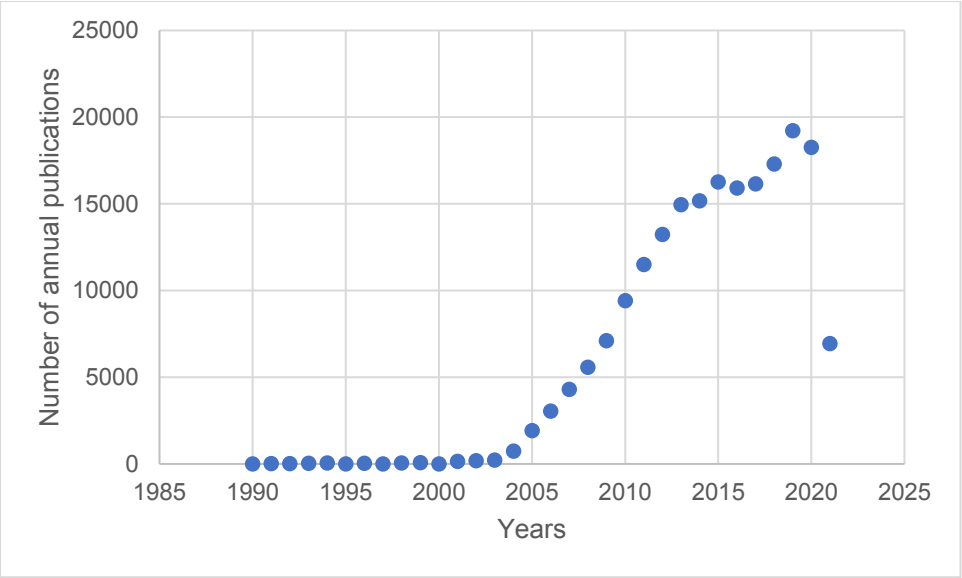

Figure 7. Summary diagram representing the number of publications per year between 1990-2021 found in PubMed database with the subject headings "nanomaterials" and "nanoparticles". The survey for the year 2021 was carried out until the month of July.

Therefore, nanotechnology is impacting several areas, providing benefits for areas such as environmental protection and remediation, engineering, biotechnology, information technology, energy, electronics, agriculture and consumer products thus contributing for the growth of the global economy (Brar *et al.*, 2010; Kong *et al.*, 2000; Lai, 2012; Maynard and Aitken, 2007). Due to their characteristics, NM have been increasingly studied for application in the biomedical field, constituting a new hope to diagnostic and therapeutic applications. Indeed, NM are expected to be used in medical imaging, chemotherapy, regenerative medicine, and as biosensors (Ramos *et al.*, 2017).

However, the same properties that make NM so unique, can also increase their reactivity and consequent interaction with the biological systems, conferring them a hazard potential which appears to be dependent on their physicochemical characteristics and that may lead to unforeseen risks to health or the environment (Aillon *et al.*, 2009; Arora *et al.*, 2012; Maynard *et al.*, 2006). As a result, this makes the biological impact of NM highly unpredictable. Thus, despite the growing interest in the use of these materials and the efforts made so far to do so, currently their applications cannot be fully explored due to the uncertainty regarding NM safety and their unknown effects on human health (Beddoes *et al.*, 2015).

Therefore, with the surprising development of nanotechnology and, consequently, the application of new NM in commercial products, human exposure to this type of material in your daily life is inevitable (Arora *et al.*, 2012; Lai, 2012; Malakar *et al.*, 2021). Human exposure to NM can occur due to the environmental pollution, the use of equipment or

products containing these materials or occupationally, the latter being a primary concern due to the production of NM by humans (Kuhlbusch *et al.*, 2018). Exposure can also occurs involuntarily, through human contact with products containing these materials, or voluntarily, in the context of its use, for instance in biomedical products (Li *et al.*, 2015). Thus, it is essential to carry out an accurate NM safety assessment, as there is an urgent need for a comprehensive understanding of its hazard because of the large spectrum of NM variants with characteristics that require testing for risk assessment (Donaldson and Poland, 2013).

1.2. Metal-based nanoparticles (M-NP): properties and applications

Various types of metals and metal oxides, such as those of silver (Ag), gold (Au), platinum (Pt), cadmium (Cd) zinc oxide (ZnO), cerium oxide (CeO_2), titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2), among others, are used for the synthesis of NP and are collectively designated as metal nanoparticles (M-NP). The application of M-NP is continually increasing worldwide in different fields such as materials science, physics, chemistry, biomedicine, and related disciplines (Jamkhande *et al.*, 2019). M-NP are available and have excellent properties for use in a variety of applications, and thus are widely used today in various consumer and industrial products (Saratale *et al.*, 2018; Schrand *et al.*, 2010).

According to the Project on Emerging Nanotechnologies (PEN; https://www.nanotechproject.tech/cpi/, accessed in June 2021), there are currently hundreds of products that contain NM, where M-NP, such as Ag, titanium (Ti), zinc (Zn), cerium (Ce), aluminium (Al), Au, iron (Fe), lead (Pb), copper (Cu), lithium (Li), carbon (C), among others, are highly represented. According to this inventory, 443 products containing AgNP, 93 with TiO_2NP and 25 with AuNP are currently available on the market. These numbers emphasize the risk of human exposure to this class of NM that seem to be associated to some degree of toxicity (Medici *et al.*, 2021).

1.2.1. Silver nanoparticles (AgNP)

For several centuries, Ag has been used as an antimicrobial agent, with silver nitrate ($AgNO_3$) being an example of a compound applied since the 17th century as a fundamental medicine for various purposes (Behra *et al.*, 2013; Klasen, 2000). The first topical application of $AgNO_3$ was probably for the healing of chronic wounds and ulcers. In the 19th century, $AgNO_3$ was also popular to treat burns (Klasen, 2000).

With the development of nanotechnology, the application of AgNP as antibacterial agents attracted much interest from various industrial sectors and, therefore, their use in

commercial products is increasing, meaning that humans are exposed to this M-NP through various products (Antony *et al.*, 2015; Calderón-Jiménez *et al.*, 2017; Lima *et al.*, 2012).

Thus, AgNP are considered as a superior product to be a manufactured in nanotechnology for use in a wide variety of consumer, biomedical, industrial, and technological applications (Behra *et al.*, 2013; Burdușel *et al.*, 2018). For instance, AgNP are used in fabrics, medical devices, cosmetics, water disinfection systems, packaging materials and in food preservation (Behra *et al.*, 2013; Reidy *et al.*, 2013).

Despite the advantages of using AgNP in several applications, their possible environmental and health effects have been the subject of current concern. Given its large-scale use, it is likely that many people are exposed to these M-NP on a regular basis, without a clear knowledge of their fate and specific behaviour in biological systems (Behra *et al.*, 2013; Lima *et al.*, 2012; Martirosyan *et al.*, 2014).

1.2.2. Gold nanoparticles (AuNP)

Au in its native form has long been regarded as chemically inert, with Au-based compounds being widely used as therapeutic agents, for example, as anti-inflammatory agents (Alkilany and Murphy, 2010; Khlebtsov and Dykman, 2011; Lopez-Chaves *et al.*, 2018).

Accordingly, AuNP also began to be actively explored in various fields of nanomedicine, for several diagnostic and therapeutic applications (Alkilany and Murphy, 2010; Khlebtsov and Dykman, 2011). In this context, AuNP can be used as carriers for drug delivery, or for the treatment of some diseases (Khlebtsov and Dykman, 2011). Although AuNP are also used in products such as cosmetics, food packaging, drinks, electronic components, and sensors, currently, these materials are more utilized in the field of medicine, such as in the diagnostics (Fröhlich and Roblegg, 2012; Lopez-Chaves *et al.*, 2018).

1.2.3. Titanium dioxide nanoparticles (TiO_2NP)

TiO_2 is an oxide that occurs naturally in three different crystalline structures: anatase, rutile or a mixture of the two (Heringa *et al.*, 2016; Peters *et al.*, 2014). Anatase is well known for its high photocatalytic activity, which makes it attractive for industrial applications. On the other hand, rutile is used as a white pigment in paints, in the rubber industry, in cosmetics (including toothpastes and sunscreens) due to its chemically inert nature (Carp *et al.*, 2004; Gerloff *et al.*, 2012; Peters *et al.*, 2014). TiO_2 is also used in everyday products such as various building materials, coatings, pharmaceuticals, and plastics. Furthermore, it

should be noted that TiO_2 is commonly used as a particulate additive in a variety of food products (e.g., chewing gum, chocolate, and candy) (Heringa *et al.*, 2016; Koeneman *et al.*, 2010; Peters *et al.*, 2014). Despite being authorized in some European Union countries under the number E171, in May 2021, following an EU request of March 2020, the European Food Safety Authority (EFSA) published a new assessment report for this food additive stating that carcinogenic effects cannot be excluded so it can no longer be considered safe.

More recently, studies have started to be carried out to expand TiO_2 applications at the nanoscale. Due to the ease of obtaining in nanometric sizes as well as for its low costs, TiO_2NP represent one of the most widely manufactured NM on a global scale (Aengenheister *et al.*, 2019; Ammendolia *et al.*, 2017).

In ready-to-use preparations, TiO_2 (nano) is allowed at a maximum concentration of 25%, except in applications that can lead to lung exposure of the end user's by inhalation (Regulation, 2019). Thus, TiO_2NP levels of different products vary over a wide range of 1 to 90 µg/mL in agents applied topically such as sunscreens and values >100 µg/mL in some paints (Tucci *et al.*, 2013).

1.3. Toxicity of metal-based nanoparticles (M-NP)

Although major advances have been made in the worldwide production and use of M-NP, there is still a serious lack of information about their impact on the environment and human health, especially on their potential for toxicity.

In addition to the characteristics of the NP, the persistence of the materials must also be considered, as this can influence the toxicity of a given material. If it is persistent, it can accumulate in the organs and inside the cells, being able to interact with cellular components and cause harmful effects to the cell. For biodegradable materials, toxicity may be associated to the toxic degradation products that are formed (Aillon *et al.*, 2009).

There are several routes of entry that can lead to human exposure to NM, among which are the dermal, ocular, respiratory (inhalation) or gastrointestinal (through ingestion) routes, in addition to injection and implantation of drug delivery systems, and these materials can then accumulate in the tissues and organs and interact with them (Beddoes *et al.*, 2015; Karmakar *et al.*, 2014; Li *et al.*, 2015; Pattan and Kaul, 2014). Therefore, the portal of entry of NM into the body is of critical importance for their putative effects (Pietroiusti *et al.*, 2013). Inhalation and ingestion are the two main pathways of NM uptake by the human body. When inhaling NM, defence mechanisms may not be able to provide complete protection against their entry. Through the airways, transport to the lungs or to the cardiovascular system can also occur, where they can remain for a long time, with a high probability of the occurrence of health problems. When in contact with the intestine, NM

may be able to cross it, reach the circulatory system, and consequently promptly reach other organs, such as the liver, spleen, and placenta (Beddoes *et al.*, 2015). NM can also enter the human body through the dermal system, and their ability to pass through the upper protective layer of the epidermis depends on its characteristics, such as size, surface chemistry and shape. Thus, depending also on skin conditions, NM can cross the protective barrier of the skin (Hagens *et al.*, 2007). As for the ocular system, when NM contact the eyes, they can cause temporary irritation and redness (Beddoes *et al.*, 2015).

In this sense, concern about the possible negative impacts of the development of nanotechnology on the environment and public health has increased (Buzea *et al.*, 2007). However, these effects are not yet fully elucidated. Thus, it is imperative to assess the toxic effects of each type of NM and to determine its limits of occupational exposure (Choi *et al.*, 2009). Considering the rapid growth of NM production and high number of NM applications, it is important to evaluate the potential toxicity that these materials might cause due to the interaction with biological systems and interference with cellular processes to understand what interactions may occur, since the possible risks for human health and the environment caused by exposure to different concentration levels of NM are not well established.

Among the various factors that can influence the biological activity and biokinetics of NM, are included characteristics such as composition, size, morphology, surface chemistry, etc. (Aranda *et al.*, 2013). Importantly, these features can determine the ability of NM to break and/or cross the biological barriers (Pietroiusti *et al.*, 2013).

Nanotoxicology is an important area of research that has emerged as a response to the need to understand and bridge gaps in knowledge of the mechanisms associated with the toxicity of NM.

There are several mechanisms by which NM can induce their toxic effects. Upon entering organisms, NM can induce cellular damage, with uptake being considered a key mechanism for their toxicity (Buchman *et al.*, 2019; Pietroiusti *et al.*, 2014). This ability is mainly due to the small size of the NM, which allows them to cross biological barriers and enter the circulatory and lymphatic systems of exposed individuals, as previously reported in humans and animals (Augustine *et al.*, 2020; Buzea *et al.*, 2007).

Once inside the cells, they can influence basic cellular processes (e.g., proliferation, metabolism, and death), alter or interrupt normal cell function when interacting with organelles and can also give rise to reactive oxygen species (ROS) (Buchman *et al.*, 2019; Pietroiusti *et al.*, 2014). Though ROS production is a normal process within the cellular metabolism, once in excessive levels, unwanted effects can occur in biological systems, such as lipid peroxidation, damage to proteins and deoxyribonucleic acid (DNA), cell death, genotoxicity and inflammatory responses (Pizzino *et al.*, 2017). Several studies have reported that M-NP induce oxidative stress. For example, Schlinkert *et al.* (2015) showed

that exposure to AuNP induced the production of ROS in human lung cells. However, it is still uncertain which characteristics of the NM might contribute to ROS production (Nel *et al.*, 2006; Schrand *et al.*, 2010).

Furthermore, as described in the review by Moreira *et al.* (2021), there is evidence that NM can induce epigenetic changes, which can trigger alterations in cellular patterns of DNA methylation, post-transcriptional histone changes and changes in non-coding RNA expression. The modified genes are mainly involved in the regulation of the epigenetic machinery itself, as well as in apoptosis, cell cycle, DNA repair and inflammation-related pathways, whose long-term changes can lead to the appearance or progression of certain pathologies, such as allergy, fibrosis, organ failure, nephrotoxicity, hepatological toxicity, splenic toxicity, pulmonary toxicity, among others (Moreira *et al.*, 2021).

AgNP are amongst the most studied M-NP. The effects of AgNP on cell function are mainly due to their size, hydrophobicity, surface modification and shape (Gioria *et al.*, 2018). Even so, although toxic effects from exposure to AgNP have already been demonstrated in several organisms, many questions about their specific interactions with the organisms at the biochemical and cellular level are still unclear. When released to the environment, AgNP may not undergo any change, interacting with the environment as M-NP; on the other hand, in the presence of moisture, AgNP can undergo oxidation, which results in the release of Ag^+ ions. Thus, the effects of these NP upon the biological systems can be mediated by its nanoparticulate and/or ion form (Behra *et al.*, 2013). When exposure to AgNP occurs for long periods and in excessive amounts (either by inhalation or by ingestion), it is reported that there may be deposition of Ag in the eyes and on the skin, with their surfaces becoming greyish in colour, a condition called argyria (Jung *et al.*, 2017).

Although it is believed that AuNP, like bulk Au, are relatively non-cytotoxic, also with these NP questions were raised regarding their potential toxicity to organisms (Khlebtsov and Dykman, 2011). However, although there are several studies that evaluate the toxicity of AuNP, the data found in the literature regarding their cytotoxicity are quite conflicting. Mesquita *et al.* (2017) examined the acute and developmental toxicity attributed to a commercial suspension of Au nanorods using early embryonic stages of zebrafish. Embryos were exposed to concentrations between 0 and 150 µg/L to determine development up to 96 h after fertilization and lethality. At sublethal concentrations, suspensions produced developmental abnormalities such as tail deformities, pericardial edema, decreased body length, and delay in eye, head, and tail elongation. On the other hand, no marked DNA damage was detected in embryos after exposure. In another study, carried out by Rattanapinyopituk *et al.* (2014), the possible route of AuNP translocation across the maternal-fetal barrier was determined, as well as the toxicity of intravenously administered AuNP in the placenta and fetus. Pregnant mice were injected intravenously with 20 and 50

nm AuNP solutions on the 16th and 17th days of gestation. There was no sign of toxic damage to placenta as well as maternal and fetal organs from the NP-treated mice.

Regarding TiO_2NP, concerns have been raised regarding the potential toxicity of these nano sized particles, especially after oral exposure (Peters *et al.*, 2014). In fact, several *in vitro* and *in vivo* studies have demonstrated that TiO_2NP can induce toxicity (Gerloff *et al.*, 2012; Johnston *et al.*, 2009). For example, Pedata *et al.* (2019) carried out a study to investigate the mechanisms by which TiO_2NP might affect the physiological function of the intestinal epithelial layer. These authors showed that exposure for 24 h to 42 µg/mL of TiO_2NP had broad effects on the integrity of Caco-2 cells, a model of the intestinal barrier.

1.3.1. Role of the physicochemical properties

The physicochemical characteristics of NM include their size and size distribution, surface area, chemical composition (e.g. crystallinity, electronic properties, purity, etc.), surface charge, dissolution, surface chemistry (surface groups, etc.), dispersion, surface roughness, porosity, solubility, aggregation, and morphology (Gatoo *et al.*, 2014).

Chemical composition is a characteristic that can influence the reactivity of NM, and therefore their intrinsic toxicity. Some studies have already addressed the influence of NM chemical composition for its toxicity. In this regard, Mortensen *et al.* (2020) carried out a study with twenty metal, metal oxide and metal sulfide NM to investigate their potential toxicity and effects upon intestinal integrity. In this study, intestinal epithelial Caco-2 cells were exposed to the selected NM for 24 h. The results demonstrated that while most of the NM studied did not adversely affect the integrity of the cell monolayer or induce cytotoxicity in Caco-2 cells, others seem to compromise intestinal integrity. The evaluation of the apparent permeability coefficient demonstrated that there was a significant increase after exposure to 15.8% (w/w) % silver-silica NP ($AgSiO_2$NP), 60 nm cadmium sulfide NP (CdS NP), 50 nm copper oxide NP (CuO NP) and 50 nm zinc oxide NP (ZnO NP), which suggests that these were the more internalized NP, contrasting with the magnesium oxide NP (MgO) that was the less internalized NM tested.

The size is an important NM physicochemical feature since is one of the main determinants of toxicity, first, due to the NM surface area that is larger for smaller particles and, second, since the body responds, distributes and eliminates materials differently depending on their size (Landsiedel *et al.*, 2012). The size makes NM chemically more reactive, but also makes them more prone to enter cells easily, exerting their potentially damaging action in places not reached by larger particles (Pietroiusti *et al.*, 2018). Thus, size may influence cell uptake, namely the mode of endocytosis and efficiency of particle

processing within the endocytic pathway(s), creating the opportunity for greater uptake and interaction with biological tissues (Aillon *et al.*, 2009; Gaumet *et al.*, 2009; Nel *et al.*, 2006). The study by Young-Man *et al.* (2018) aimed to assess the acute toxicity of AgNP with various sizes (10, 60 and 100 nm) administered intraperitoneally in mice. It was observed that all 10 rats administered with 10 nm AgNP were found dead or moribund within 24 h. On the other hand, these effects were not evident after the administration of 60 and 100 nm AgNP. The results of this study then suggest that AgNP that are smaller in size have significantly higher acute toxicity in mice.

NM chemical constitution and crystalline structure can influence the toxicity of a given material (Buzea *et al.*, 2007). For example, Gerloff *et al.* (2012) evaluated the toxicity of TiO_2NP with different chemical structures, using samples containing rutile-anatase and pure anatase. The potential of these TiO_2NP (20 and 80 μg/cm^2) to induce cytotoxicity, oxidative stress and DNA damage was evaluated in human intestinal Caco-2 cells. Only rutile-anatase TiO_2NP but not anatase TiO_2NP induced significant LDH release and mild DNA damage in Caco-2 cells. However, none of the tested TiO_2NP seem to affect cellular biomarkers of oxidative stress. Therefore, these results suggest that rutile-anatase TiO_2NP are more toxic than anatase TiO_2NP, supporting the fact that chemical structure is an important driver of NM toxicity.

Currently, the NM toxicity is being addressed by a series of approaches that involve *in vitro* and *in vivo* studies, including detailed genomics or biodistribution studies.

1.3.2. *In vitro* studies

There is a growing incentive to reduce animal testing and develop more physiologically relevant *in vitro* models. With the large number of available NM already in use in consumer products whose safety needs to be assessed, it is important to align nanotoxicology studies with the 3Rs principles (replacement, reduction, and refinement of animal testing) (Mortensen *et al.*, 2020). Thus, since evaluating all the existing NM on a case-by-case basis is both expensive and time-consuming, alternative *in vitro* models are extremely important to accelerate NM testing (Fröhlich, 2018). In this context, a sensitive point is that *in vitro* conditions not always mimic the *in vivo*, real-life situation, which can difficult data interpretation and human translation.

Another aspect of particular importance is the rather common interference of the NM with the reagents/components of the *in vitro* assays that can compromise the reliability of the test and difficult the interpretation of data (Kroll *et al.*, 2012). In addition to allowing quick and easy measurements to be performed, *in vitro* studies provide a vital tool that allows to establish toxicity mechanisms associated with a given material, providing estimates for

hazard ranking. Thus, these studies help to decrease the number of *ex vivo* or *in vivo* testing (Carreira *et al.*, 2015; Kang *et al.*, 2013).

Despite the various *in vitro* studies already carried out to determine the safety of NM, the conclusions drawn are often inconsistent, since the materials and conditions used in different tests are different, making it difficult to compare results between studies.

Ahamed *et al.* (2008) evaluated the toxicity of 25 nm AgNP uncoated- and polysaccharide-coated in mouse embryonic stem cells and fibroblasts. Induction of cell death by both types of AgNP was observed, however some differences have been detected, namely in DNA damage, where the polysaccharide coated AgNP induced more severe damage than the uncoated NP. These results suggest that the coated NP are more evenly distributed compared to uncoated NP that seem to aggregate/agglomerate, limiting the surface area availability and access to the membrane-bound organelles.

Different *in vitro* studies that evaluated the toxicity of AuNP showed different results regarding the toxicity induced by these materials. Ponti *et al.* (2009) evaluated the cytotoxicity of 8 nm AuNP in MDCK and HepG2 cells and found that these NP were not toxic during a 24-hour exposure period. In contrast, Pan *et al.* (2007) observed size-dependent toxicity of AuNP in four cell lines (connective tissue fibroblasts, epithelial cells, macrophages and melanoma cells). In this study, 0.8, 1.2, 1.4, 1.8 and 15 nm AuNP were tested and the results showed that 1.4 nm AuNP were 60-fold more toxic than 15 nm AuNP (Pan *et al.*, 2007)

Regarding TiO_2NP, Zhang *et al.* (2013) showed that these NP caused a low toxicity in rat macrophages exposed to different concentrations (12.5, 25, 50, 100, 200, 400 and 600 µg/mL). Even so, they were able to observe differences between cytotoxicity and oxidative stress caused by TiO_2NP of different sizes and crystal structure: three types of anatase TiO_2NP (with 5, 25 and 100 nm) and a rutile TiO_2NP (with 100 nm). The particles that induced more cytotoxicity and oxidative stress were 25 nm anatase TiO_2NP, followed by the 5 and 100 nm anatase TiO_2NP. On the other hand, 100 nm rutile TiO_2NP where the ones that induced the lower level of toxicity. Based on these results, they concluded that the structural characteristics of TiO_2NP may be the main factor related to their toxicity. Dorier *et al.* (2019) also investigated the *in vitro* impact of the food additive E171 and TiO_2NP on a co-culture of intestinal Caco-2 and HT29-MTX cells. Co-cultures were exposed for 6, 24 and 48h to 10, 50, 100 and 200 µg/mL of both particles. Exposure of the cells to TiO_2 particles resulted in increased levels of intracellular ROS in a concentration-dependent manner for E171, however, it did no impair cell viability, nor did it cause oxidative DNA damage or stress on the endoplasmic reticulum. Thus, the study showed that the food additive E171 and TiO_2NP produce only minor effects in the tested *in vitro* intestinal cell model.

1.3.3. *In vivo* studies

Considering the complexity of NM and the numberless characteristics that can contribute to their toxicity, their characterization *in vivo* becomes a very complicated task.

Nevertheless, several studies have already been conducted to test NM toxicity *in vivo*. In this regard, Cha *et al.* (2008) fed mice with 13 nm AgNP or 2 - 3.5 μm Ag microparticles. In these animals, livers were collected 3 days after exposure and submitted to histopathological analysis. The results showed signs of liver inflammation, which means that these NP had a harmful effect in these mice.

For AuNP, most of the *in vivo* studies focused on the acute effects, biokinetics and distribution of these NP, namely the effect of size and shape. According to a study by Lopez-Chaves *et al.* (2018) in Wistar rats exposed by 50 mg/L to 10, 30 or 60 nm AuNP, Au measurements in the liver and kidney showed that rats exposed to 10 and 30 nm AuNP accumulated the highest levels, showing no significant differences between the two groups. Lower Au content was found in the group treated with 60 nm AuNP. Interestingly, a very strong presence of 10 nm AuNP was found in the intestinal tissue. This study showed that AuNP are distributed according to size, and that smaller NP are more toxic than larger ones.

Tassinari *et al.* (2014) explored the possible reproductive and endocrine effects of short-term (5-day) oral exposure to anatase TiO_2NP in rat. In the spleen of the treated animals, TiO_2NP aggregates were detected, although Ti tissue levels remained low, reflecting the short exposure time. Therefore, the results demonstrated deposition of TiO_2NP in rat internal organs, as well as reproductive and endocrine effects after short-term oral exposure to dose levels of NP consistent with real-life exposure scenarios. At the same time, Yamashita *et al.* (2011) reported that 35 nm TiO_2NP can cause complications in pregnancy when injected intravenously into pregnant female rats, since they are able to cross the placental barrier, reaching other organs such as the fetal liver and brain. Indeed, rats treated with these NP had smaller uteri and fetuses than the unexposed controls.

1.3.4. Epidemiological evidence

Many of the epidemiological studies in the field of NM relate to particle toxicity assessments of air pollution. At the end of the previous century, several epidemiological studies identified health effects induced by particulate matter (<100 nm – 2.5 μm) transported by air at levels that, at that time, were considered safe. Ultrafine particles (UFP; <100 nm) have also been identified as one of the components responsible for adverse health effects observed at acceptable external levels. Although UFP and NM are often derived from very different sources and processes, they both possess similar

physicochemical characteristics, suggesting that their properties, behaviour and, most importantly, their toxicity may also overlap (Stone *et al.*, 2017).

Even so, existing epidemiological studies show that the adverse effects of NP on human health depend not only on their physical and chemical properties, but also on the conditions of exposure (concentration of NP, frequency and duration of exposure) and individual factors, such as genetics and the pre-existence of medical conditions in the exposed individual as it seem that diseased individuals may be more susceptible to exposure to NP (Oberdörster *et al.*, 2007).

As previously stated, workplace exposure to NM is increasing. In the pilot study by Lee *et al.* (2012), workers from a AgNP manufacturing plant were assessed for their personal exposure levels. The manufacturing process was also evaluated, and blood and urine samples were taken from the exposed workers. The levels of Ag in their blood and urine were low. When the blood chemistry and haematology parameters were evaluated by an occupational physician, the blood data were determined to be within a normal range. Taken together, data on the health status of NM manufacturing workers did not show any adverse effects.

On the other hand, occupational exposure can occur in people who do not manufacture NM, but who end up being exposed to products that contain NM and that serve as input materials or to processes that generate NM that do not serve to produce or use NM. One such case is the ceramic industry, where NM such as GO, TiO_2 and SiO_2 are used, among others. The review by Bessa *et al.* (2020) highlights the importance of studying particulate emissions generated by production processes such as fine and ultrafine airborne fractions in ceramic workplaces and their impact on the worker's exposure to air. Although the number of workers in these plants are in most the available case studies not high, this article emphasizes the importance of risk assessment and the implementation of preventive and mitigation measures to improve the occupational air quality in the ceramic industries.

1.4. Impact of the nanomaterials (NM) in the biological barriers

The access of an almost infinite number of potentially harmful agents to the body is strongly limited by the defence system constituted by the biological barriers, such as the mucous, cornea, epithelial skin, gastrointestinal tract (GIT), endothelial, placental, and pulmonary air-blood and blood-brain barriers. These barriers play an important role in preventing the access of foreign substances and microorganisms to the deepest levels of organs, serving as a primary defence (Meng *et al.*, 2018).

Several reports shown that NM can pass through the biological barriers and accumulate in different organs inducing toxic effects, such as oxidative stress, DNA

damage, cell death and morphological changes, raising concerns that they may potentially affect the physiology of any cell in the body (Abudayyak *et al.*, 2017; Arora *et al.*, 2012; Ema *et al.*, 2010). The physicochemical characteristics of NM can have a different impact on biological barriers. Such interactions can affect cell compartments or biological molecules and may even promote their internalization (Meng *et al.*, 2018).

In the present dissertation, two representative cell models of the intestinal and placental barrier were studied.

1.4.1. The intestinal barrier

A healthy digestive system ensures an effective absorption of nutrients from the intestine after food digestion, but also prevents the passage of foreign materials (Ammendolia *et al.*, 2017; Mortensen *et al.*, 2020). Thus, this is a selective mucous barrier with digestive, absorbent, secretory and protective functions, whose integrity is essential to maintain (Bergin and Witzmann, 2013).

Several models have been used for decades to assess the solubility, digestion, and epithelial permeability of food and drugs along the GIT. Toxicokinetic studies in animals have historically been used to assess these parameters for conventional chemicals. However, ethical consideration and approval is necessary for the use of animals. *Ex vivo* models use tissue samples that are excised from an anesthetized organism and maintained under conditions that support certain aspects of normal function. *Ex vivo* gastrointestinal models suffer from the disadvantage of lacking digestive juices. However, they are more efficient than *in situ* studies. *In vitro* cell models are one of the several options for replacing animal experimentation. They allow screening for epithelial permeability mechanisms, potentially in a high-throughput manner. Primary epithelial cells isolated from GIT are not widely used because they have a restricted shelf life in culture and are often unable to form an organized monolayer. Effective *in vitro* systems generally use immortalized cell lines that form an adherent monolayer that reflects certain characteristics of the GIT epithelium. Several cell lines originating from different segments of the GIT are commonly used in the risk assessment of chemical substances, and, among the several available, Caco-2 intestinal cells have been the most used. They represent enterocytes, the most abundant type of epithelial cell in the intestine (Lefebvre *et al.*, 2015). This is one of the most relevant *in vitro* culture models used in the study of intestinal functions, which mirrors the human small intestine epithelium, being a well-characterized cell line that displays a faithful representation of what structural characteristics are *in vivo*, providing a powerful tool for studying the properties of the intestinal epithelium due to its simplicity and reproducibility, this way allowing a interlaboratory comparison of results (Bailey *et al.*, 1996; Koeneman *et*

al., 2010). In this study, as a way to study the toxicity of M-NP in the intestinal barrier, the Caco-2 cell line was used, a line derived from human epithelial cells from colorectal adenocarcinoma (Chen *et al.*, 2016; Kang *et al.*, 2013; Mortensen *et al.*, 2020).

Moreover, HT29 cells (originating from human colorectal adenocarcinoma) secrete mucus and provide a useful model of the adhesive and barrier properties of the gastrointestinal mucus layer (Lefebvre *et al.*, 2015; Xu *et al.*, 2021). However, monoculture does not fully reflect the human intestinal epithelium, where goblet cells represent 10-25% of cells. In addition, there are *in vitro* cell co-culture systems that integrate several cell types and human-based 3D tissue models that are commercially available and that may be applicable to NM. Recently, several high-performance *in vitro* and *in silico* models of mammalian GIT have been used, which can provide a large amount of human relevant data very quickly. There are also *in silico* programs driven by complex computational algorithms designed to predict how new substances will behave in the human GIT (Lefebvre *et al.*, 2015).

Translocation of particles across the intestinal barrier is a multi-step process that involves diffusion through the mucus layer and uptake via paracellular or transcellular transport (**Figure 2**). The NM can reach the GIT either directly by intentional ingestion or indirectly by dissolving NP from food containers or by secondary ingestion of inhaled particles (Bergin and Witzmann, 2013). This exposure can occur through the ingestion of food and water and the use of cosmetics, medications and medication delivery devices (Pattan and Kaul, 2014). Since the GIT represents a large surface area, its interactions with ingested NP are facilitated (Bergin and Witzmann, 2013).

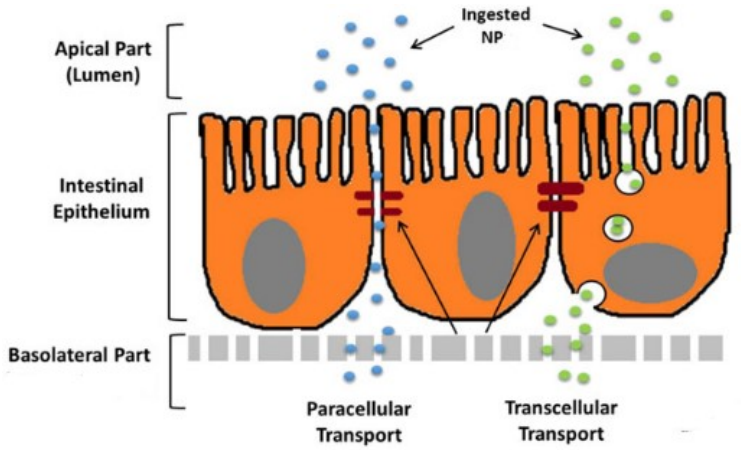

Figure 8. Scheme showing transport of nanoparticles (NP) through the intestinal barrier. Adapted from Vila *et al.* (2018).

Although the absorption of nutrients at the nanoscale by the GIT is a naturally occurring physiological process, there is evidence suggesting that the nano sized TiO_2 particles absorbed may interfere with intestinal functions in humans. Food-grade TiO_2 was found in the ileum of both healthy and ulcerative colitis afflicted children, with deposition increasing with age. TiO_2 deposits have also been reported to occur in patients with colon adenocarcinoma, Crohn's disease and non-Crohn's disease-associated colitis (Ammendolia *et al.*, 2017). However, the possible adverse effects of these NP on the intestinal epithelium are still poorly investigated.

1.4.2. The placental barrier

Among the most vulnerable populations to xenobiotics exposure are pregnant women and developing embryos/fetuses (Aengenheister *et al.*, 2019; Bongaerts *et al.*, 2020). Thus, essential during pregnancy, the placenta is a fundamental organ to support the growth and development of the embryo and fetus, being a semipermeable barrier that performs exchange functions in two directions: the transfer of nutrients, oxygen, and hormones from the mother to the fetus and the removal of waste products from the embryonic side into maternal blood; therefore, the placental barrier is a complex and vital part of the reproductive defensive systems (Meng *et al.*, 2018; Prouillac and Lecoeur, 2010). Exposure to environmental chemicals during pregnancy can be detrimental to the health of the placenta and therefore have an adverse impact on maternal and fetal health (Fry *et al.*, 2019). When exogenous substances are present in the maternal bloodstream, the extent to which this exposure affects the fetus is determined by transport processes in the placental barrier. Three layers separate the maternal and fetal blood flows, forming a physical barrier between their circulations: syncytiotrophoblasts, connective tissue and fetal vascular endothelium (Cartwright *et al.*, 2012) as depicted in **Figure 3**.

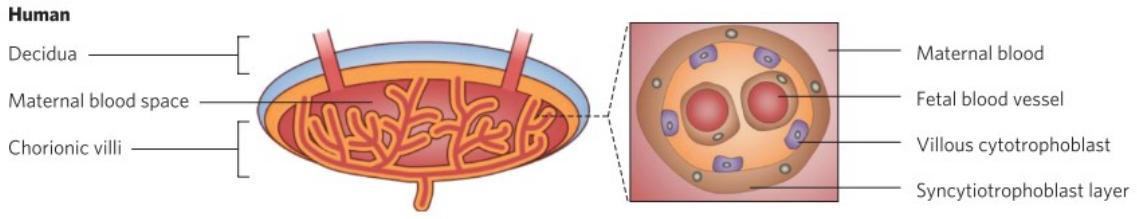

Figure 9. Schematic representation of the human placenta. Adapted from Yamashita *et al.* (2011).

To study substance translocation across the human placenta, several experimental *in vivo* and *in vitro* models were developed, including animal models, *ex vivo* perfusion of

human placenta, immortalized cell lines, primary culture cells, and villus explants cultures (Cartwright *et al.*, 2012; Moe, 1995; Prouillac and Lecoeur, 2010; Rothbauer *et al.*, 2017). The biggest problem with animal models is that the placental organ architecture has great interspecies variability. Although the *ex vivo* human placental perfusion model provides a controlled system that allows investigation on a whole organ scale with organized tissue architecture, it exhibits low reproducibility, low throughput features and no standardization. Furthermore, the main limitation of this technique is that these *ex vivo* models are limited for studies of the third trimester gestation. As an isolated organic system, placental perfusion does not naturally reflect the *in vivo* pharmacokinetic balance of the entire maternal-placental-fetal system (Myllynen *et al.*, 2013; Rothbauer *et al.*, 2017). Even though a wide range of techniques have been developed so far due to the low availability of fresh tissue samples and therefore primary cell cultures, choriocarcinoma cell lines remain a reasonable *in vitro* model for placental research, as they are easy to manipulate and propagate. Although these cells represent a model of cancer cells, they still exhibit several key human placental trophoblast capabilities, including hormone release, expression of glucose transporters, as well as barrier capacity (Rothbauer *et al.*, 2017). The human placental continuous cell lines most used in toxicology research are the oldest placental cell lines BeWo, JAR and Jeg-3, as well as the human recently established ACH-3P trophoblast cell line. BeWo cells originate from choriocarcinoma that was first transferred to a hamster's cheek pouch and later co-cultured with deciduous human explants, from which BeWo cells were isolated. The same choriocarcinoma tissues were also used to create the Jeg-3 choriocarcinoma cell line. JAR choriocarcinoma cells were established from a human placental trophoblastic tumour. Compared to the use of immortalized or cancer cell lines, establishing primary placental cell cultures is cumbersome. Once isolated, the primary villous cytotrophoblasts isolated from a single placenta can be stored frozen to be used in multiple experiments. However, isolated primary trophoblasts do not proliferate in culture, which is a major disadvantage of this model. On the other hand, primary cells clearly have the advantage of representing normal cells, whereas cancer cell lines suffered malignant transformation. Like the *in vivo* situation, villous explants contain several types of cells, in addition to trophoblastic cells, which is an advantage for this system. Unlike isolated primary trophoblasts, villous explants are not regularly frozen. Thus, for each experiment, fresh placental tissue is needed, and cultures cannot be repeated using the same placenta (Myllynen *et al.*, 2013). Studies on drug concentrations in cord blood show a reasonable correlation between *in vitro* and *in vivo* results, supporting the validity of *in vitro* models (Vinith *et al.*, 2011).

The physiological changes that occur during pregnancy can alter the biodistribution of NM, increasing the potential risks to the mother and the development of the fetus

(Aengenheister *et al.*, 2019). The pregnancy stage determines the permeability of the placental barrier, since the thickness of the syncytiotrophoblast layer decreases progressively with gestational age, gradually becoming more permeable from the first trimester to the third and, consequently, facilitating the exchange processes between the mother and the fetus. Thus, the more advanced the state of pregnancy, the greater the probability than a given NM will cross the placenta (Meng *et al.*, 2018; Prouillac and Lecoeur, 2010).

NM that infiltrate the bloodstream can reach the placenta and, as has been shown in *in vivo* and *ex vivo* studies in animals and humans, some xenobiotics (depending on the physical and chemical properties) can cross the placental barrier, passing from the mother to the fetus (Bongaerts *et al.*, 2020; Dimopoulou *et al.*, 2018). M-NP have already been found in samples of human amniotic fluids from pregnant mothers with healthy fetuses and fetuses with congenital malformations, mostly in the form of clusters, suggesting that these particles penetrate the amniotic fluid and can affect human fetuses (Barošová *et al.*, 2015). Both *in vitro* and *ex vivo*, accumulation of AuNP (Aengenheister *et al.*, 2018; Myllynen *et al.*, 2008), TiO_2NP (Aengenheister *et al.*, 2019) and SiO_2NP (Poulsen *et al.*, 2015) were observed in the human placenta. Manangama *et al.* (2019) showed a significant association between maternal occupational exposure to NP during pregnancy and an increased risk of newborns being small for gestational age (SGA).

There is an association between placental transfer and particle size, with placental transfer being increased after perfusion with smaller particle sizes. Using an *ex vivo* human placental perfusion model, Grafmüller *et al.* (2013) demonstrated that 80 nm polystyrene particles were able to cross the placental barrier, while 500 nm particles were retained in the placental tissue or maternal circuit.

As a way of assessing the impact of NM on the placental barrier, the BeWo b30 cell line, originating from a human choriocarcinoma that retains cell properties of mononucleated cytotrophoblasts, was used in the present dissertation. The BeWo b30 clone showed a better monolayer formation capability than the original BeWo clone. Although this model does not constitute a complete physiological system and the entire microenvironment found in humans, it functions as an *in vitro* model that represents the limiting barrier of maternal-fetal transfer rate. Due to the differences between species in the structure of the placenta (Cartwright *et al.*, 2012; Mathiesen *et al.*, 2014; Prouillac and Lecoeur, 2010) in this work the focus was on a model of human origin.

2. Aims

2. Aims

Owing to their appealing properties, NM have undergone a continuous expansion of manufacturing and applications in major industrial, research and biomedical fields and have attracted considerable attention, becoming a major global public health problem. However, mounting evidence suggests that certain NM are cytotoxic in cultured cell systems. Thus, also due to greater environmental and occupational exposure, an appropriate risk assessment and establishment of parameters for the safe use of these materials is essential. In recent years, the cytotoxicity of NM has been thoroughly assessed. Many economically relevant M-NP, such as AgNP and AuNP, have been investigated for their toxicological potential. Studying the possible effects of these particles on health is of great importance. Therefore, it is essential to determine the role of NM properties in their induced biological effects.

Cellular barriers are more complex *in vivo*, incorporating thin capillaries, stromal compartments, and basement membranes, as well as active transport functions, but existing *in vitro* models are still valuable for preliminary screening. Viability tests are vital steps in toxicology that explain the cellular response to a toxicant by providing information on cell death, survival, and metabolic activities. The assessment of different endpoints is important, as NM can induce toxicity by a variety of mechanisms. Therefore, to achieve the main objective of the present work, a multiparametric assessment of the in vitro toxicity of different NM was carried out.

The present investigation was designed to expand the existing knowledge about the potential toxicity of M-NP with different chemical constitution (Ag, TiO_2 and Au), size (10, 30 and 60 nm), chemical structure (rutile and anatase) and surface capping (citrate and PEGylated-AuNP). For the sake of comparison, non-metallic NM were also tested, namely SiO_2NP and nano-graphene oxide (nano_GO).

The *in vitro* toxicological assessment was carried out on two human models of epithelial barrier cells:

- Caco-2 cell line, consisting of cells of the human intestine, which represents the intestinal barrier.
- BeWo clone b30 cell line, consisting of human trophoblastic cells, which represents the placental barrier.

These are well-characterized and relevant models for the study of NP toxicity, as they constitute a fundamental line of defence against substances and foreign bodies. To make this assessment, several parameters were studied that include cell morphology, membrane integrity, metabolic capacity, oxidative stress (ROS levels) and ATP levels.

Therefore, this study aimed to:

1) Identify the main mechanisms of *in vitro* toxicity of different M-NP in two cell models of biological barriers;
2) Understand the influence of M-NP chemical composition, size, crystalline structure, and surface coating for the observed effects;
3) Determine whether the observed cytotoxic effects induced by the selected M-NP are specific to each cell barrier.

The results to be obtained are expected to contribute to increasing knowledge of the potential harmful effects of NM, with a special focus on M-NP on human intestinal and placental biological barriers. Understanding the toxicity of these NM would assist scientific progress, contributing to greater effectiveness in assessing the growing number of NM. Thus, it can guide the development of a safer and more sustainable nanotechnology industry, reducing the adverse effects of these NM in the future.

3. Materials & Methods

3.1. Nanomaterials (NM) and chemicals

3.2. Methods

3.2.1. Cell culture

3.2.2. Handling and preparation of the nanomaterials (NM) suspensions

3.2.3. Cellular exposure to the nanomaterials (NM) suspensions

3.2.4. *In vitro* general toxicity assessment tests

3.2.5. Detection of reactive oxygen species (ROS) generation

3.2.6. Determination of intracellular ATP levels

3.3. Statistical analysis

3. Materials & Methods

The laboratory work was carried out in accordance with INSA safety guidelines, with the use of personal protective equipment (namely, lab coat and gloves). All the disposable material that was in contact with any toxic compound as well as hazardous wastes were disposed in suitable containers.

3.1. Nanomaterials (NM) and chemicals

Differently sized BioPure™ AgNP and AuNP (citrate-coated 10, 30 and 60 nm), BioPure™ AuNP_10_PEG and NanoXact™ 20 nm SiO_2NP were purchased from nanoComposix, Inc (San Diego, CA, USA). The nano_GO and TiO_2NP_anatase were obtained at Sigma-Aldrich®; TiO_2_NM105 (rutile-anatase) were obtained from the Joint Research Centre (JRC) repository. Dulbecco's modified Eagle's medium (DMEM; # P04-05550), fetal bovine serum (FBS), penicillin-streptomycin (# P06-07100) and 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) solution were purchased from PAN Biotech (Aidenbach, Germany). $AgNO_3$, Triton X-100, Roche Lactate dehydrogenase (LDH) cytotoxicity detection kit were purchased from Sigma-Aldrich Co. (Madrid, Spain). Ethanol (EtOH) was obtained at VWR (Fontenay-sous-Bois, France) and phosphate buffered saline (PBS 10×) at BioWittaker®. Lactate dehydrogenase (LDH) cytotoxicity detection kit was obtained from Roche. Invitrogen™ AlamarBlue, CM-H_2DCFDA (ROS indicator) and adenosine triphosphate (ATP) luminescence kit were obtained from ThermoFisher (Madrid, Spain).

3.2. Methods

3.2.1. Cell culture

The cytotoxicity testing was performed in cells that represent the intestinal and placental barriers, in the human colorectal adenocarcinoma Caco-2 cell line (ATCC®, Cat. #HTB37™, Lot. #70013347; Manassas, VA, USA) and in the human placental choriocarcinoma BeWo b30 cell line (AddexBio, Cat. #C0030002, Lot. #7985832; San Diego, CA, USA). Both cell lines have an epithelial morphology. Briefly, Caco-2 and BeWo b30 cells were grown in flasks as monolayer cultures in culture medium (DMEM) containing 4.5 g/L glucose, 4 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate and 3.7 g/L sodium bicarbonate ($NaHCO_3$)

supplemented with 100 units/mL penicillin and 0.1 mg/mL streptomycin, and 20% and 10% (v/v) of FBS, respectively.

Cells were kept in cell culture flasks in an incubator at 37ºC in a humidified atmosphere of 5% carbon dioxide (CO_2) (Binder GmbH, Tuttlingen, Germany). Cells were subcultured by removing cell culture medium from the flasks and washing the cells with a 0.25% trypsin- EDTA solution. After trypsin-EDTA solution removal, fresh trypsin-EDTA solution was added, and the cells were incubated at 37ºC for approximately 5-7 minutes until all cells detached from the flask. To inactivate the trypsin-EDTA solution, at least two times the volume of trypsin-EDTA of cell culture medium was added to the flask. Then, the cells were seeded at a density of approximately 1.2×10^4 cells/cm^2 and 9.3×10^3 cells/cm^2 for BeWo b30 and Caco-2 cells, respectively. Cell culture medium was replaced every 2/3 days. The cells were passed once a week. Cells were cultured to maximum of ~15 passages to avoid changes in their properties associated with a high number of passages (Bailey *et al.*, 1996). Both the cell culture medium and the trypsin-EDTA solution were stored at 4ºC.

3.2.2. Handling and preparation of the nanomaterials (NM) suspensions

Table 1 summarizes the main physical and chemical properties of NM. All procedures of handling and preparation of the MN were standardized to minimize within-experiment variations. All the tested NM were freshly prepared under sterile conditions and diluted in serum-free cell culture medium directly before application. Nano_GO, rutile-anatase and anatase TiO_2NP were dispersed in serum-free cell culture medium (0.5 mg/mL) by indirect probe sonication using a Bandelin Cup Horn (Berlin, Germany) according to the internal Standard Operating Procedure (SOP) of NanoToxClass project consortium (NanoToxClass, 2017).

SiO_2NP aqueous suspension was sonicated in an ultrasound bath for 5 min and Ag- and AuNP briefly vortexed before dilution in the serum-free cell culture medium, as recommended by the manufacturer.

Table 1. Main physicochemical characteristics of the nanomaterials (NM) under study.

Aqueous suspensions

		Parameter							
Nanomaterial	**Provider**	**Particle surface**	**Primary particle size (nm)**	**z-average size (nm)**	**Surface area (m^2/g)**	**Zeta potential (ZP)@ pH 7.4 (mV)**	**pH**	**Particle concentration (particles /mL)**	**Mass concentration (mg/mL)**
AgNP_10	nanoComposix	citrate	10.3 ± 1.9	14	51.9	-28	8.1	1.7×10^{14}	0.99
AgNP_30	nanoComposix	citrate	30 ± 3	34	18.5	-44	7.9	7.1×10^{12}	1.08
AgNP_60	nanoComposix	citrate	60 ± 7	64	9.3	-46	7.6	9×10^{11}	1.08
AuNP_10	nanoComposix	citrate	10.4 ± 0.9	12	29.5	-33	5.6	9.5×10^{13}	1.08
AuNP_30	nanoComposix	citrate	30 ± 3	37	10.2	-56	6.8	4.1×10^{12}	1.09
AuNP_60	nanoComposix	citrate	61 ±5	72	5	-61	6.9	4.6×10^{11}	1.06
AuNP_10_PEG	nanoComposix	mPEG 5 kDa	8.6 ± 0.8	Not reported*	35.8	Not reported*	5.2	1.7×10^{14}	1.10
SiO2	nanoComposix	Silanol	21.6 ± 2.1	20	124	-34	8.3	4.8×10^{14}	5.6

*DLS and zeta potential not reported; the concentration of the suspension is too low to get sufficient scattered light for an accurate measurement.

Powders

Nanomaterial	**Provider**	**Crystal Structure**	**Particle surface**	**Primary particle size (nm)**
TiO_2_anatase	Sigma-Aldrich	anatase	none	<25
TiO_2_NM105	JRC	rutlie-anatase	none	<25

Nanomaterial	**Provider**	**Material surface**	**Number of sheets**
nano_GO	Sigma-Aldrich	4-10% edge oxidized	10-20 sheets

3.2.3. Cellular exposure to the nanomaterials (NM) suspensions

The experimental design of the toxicity testing of the experiments is depicted in **Figure 4**. The tests were carried out at 6 days post-seeding. On day one, the cells were seeded; after 24 h, cell culture medium was replaced by fresh medium and cells let grow for the next 2 days; on the fifth day, the cells were exposed to the tested NM and, on the last day (day 6), the *in vitro* assays were performed.

Figure 10. Timeline and experimental design of the *in vitro* studies.

For the experiments, after detaching the cells from the culture flasks at 80-90% confluence, cells were counted in a hemocytometer. For each toxicological assay, cells were plated at a density of 3.03×10^4 cells/cm^2 for Caco-2 cells and 6.06×10^4 cells/cm^2 for BeWo b30 cells (100 µL cell suspension per well) in 96-well cell culture plates (diameter = 0.33 cm) and let grow for 4 days. At confluence, cells were exposed for 24 h to varied concentrations (0.8, 1.5, 3, 6, 12, 24 and 48 µg/cm^2 of the M-NP (AgNP, TiO_2 NP and AuNP) and non-metallic NM (SiO_2NP and nano_GO). Unexposed cells served as negative control (NC); suitable positive controls (PC) were also included in all assays. At the end of the exposure period, cells were monitored for morphological changes under an optical microscope (VWR International).

3.2.4. *In vitro* general toxicity assessment tests

Cells were seeded in 96-well plates and incubated with increasing concentrations (0-48 µg/cm^2; 100 µL) of the test NM during 24 h at 37 ºC, 5% CO_2. Two cytotoxicity endpoints were determined: AlamarBlue (resazurin) reduction and lactate dehydrogenase (LDH) release, indicators of metabolic activity and plasma membrane integrity, respectively. Cell metabolic activity was evaluated using AlamarBlue reagent, according to the manufacturer's instructions. Cells exposed to 70% ethanol (30 min) were used as PC. After exposure, the incubation medium was removed and cells incubated with 100 µL/well of AlamarBlue reagent diluted 1:10 in serum-free cell culture medium for 4 h at 37 ºC, 5% CO_2. Sample's fluorescence was measured at 570/610 nm (excitation/emission) in a microplate reader (Molecular Devices SpectraMax® iD3). Fluorescence values were normalized considering the NC mean value.

LDH release was determined using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany), according to manufacturer's instructions. Following the exposure period, incubation media were gently pipetted to a 96-well round bottom microplate and centrifuged for 5 min at 2000 *x g* to remove the cell debris and residual NM. Cells lysed with 0.2 % Triton X-100 (30 min) were used as the PC. Briefly, 100 µL of freshly prepared reaction mixture was added to 100 µL of each sample and incubated up to 30 min at 15-25 ºC (protected from light). Absorbance was measured at 490 nm and 630 nm (reference wavelength) in a microplate reader (Molecular Devices SpectraMax® iD3). LDH release values were normalized considering the PC mean value (total LDH release). Triton X-100 (0.2 %) was taken as PC and set as 100% cytotoxicity. The values obtained thereafter have been expressed as a percentage compared to PC, [(optical density $(OD)_{sample}$-OD_{blank})/(OD_{PC}-OD_{blank}) x 100].

3.2.5. Detection of ROS generation

Intracellular ROS levels were detected using a ROS-sensitive fluorescent probe, the carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-DCFH-DA), according to the manufacturer's guidelines. Carboxy-DCFH-DA is non-fluorescent, unless oxidized by intracellular ROS.

To detect the generation of ROS, cells were seeded in 96-well plates and grow to confluence and incubated with 10 µM carboxy-DCFH-DA probe (100 µL/well) of serum-free medium for 1 h at 37ºC, 5% CO_2. At the end of this period, the medium was removed, and the cells exposed to the selected concentrations of the NM under investigation (0.8 - 12 µg/cm^2). After incubating the cells for 24 h, the fluorescence of 2',7'-dichlorofluorescein (DCF) was measured in a microplate reader (Molecular Devices SpectraMax® iD3) at 485-492 / 520-530 nm (excitation/emission). Cells incubated without NM were used as a NC, whereas cells exposed to $AgNO_3$ served as PC (0.38 and 0.75 µg/cm^2). Fluorescence values were normalized considering the NC mean value.

3.2.6. Determination of intracellular ATP levels

The intracellular ATP levels were measured using the ATP Determination kit (Molecular Probes; Invitrogen) according to the manufacturer's recommendations. Cells exposed to 10 mM sodium fluoride (NaF) served as PC of this assay. Following the exposure period, cells were lysed with 200 µL of Passive Lysis Buffer (Promega) and then centrifuged for 5 min at 2200 *x g* to remove the cell debris and residual NM. To measure the intracellular ATP content, 10 µL of each sample/ATP standards were pipetted into 96-

well white microplates (Perkin Elmer) and 90 µL of reaction buffer added to each well. Luminescence was measured in a microplate reader (Molecular Devices SpectraMax® iD3) with 1 s of integration time. The background luminescence was subtracted from the measurements. ATP concentrations were calculated from the ATP standard curve calculated for each 96-well microplate assay. Total ATP was normalized by the number of cells per well, estimated by measuring each sample nuclei acid content, using propidium iodide (PI). For this purpose, the samples were incubated with a 2 µg/mL PI and fluorescence read at 530/620 nm (Excitation/Emission) in a microplate reader (Molecular Devices SpectraMax® iD3).

3.3. Statistical analysis

Results are expressed as mean ± standard deviation (SD) of two-three independent experiments. Statistical analyses of data were performed using GraphPad Prism 6 for Windows (La Jolla, CA, USA). Data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively. Data were analysed by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett's test for multiple comparisons to test for differences between control and exposed cells. Significance was accepted at a P value <0.05.

4. Results and Discussion

4.1. Intestinal barrier (Caco-2 cells)

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4.2. Placental barrier (BeWo b30 cells)

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4.3. Intestinal versus placental barrier

4. Results and Discussion

4.1. Intestinal barrier (Caco-2 cells)

4.1.1. Monitoring of cell morphological changes by optical microscopy

Figure 5 depicts the morphological aspect of Caco-2 cells upon 24 h of exposure to the tested NM. Although morphological changes are not visible in cells exposed to all tested NM, it is possible to observe aggregation/agglomeration of some NM, with the formation of large clusters, for instance in SiO_2NP-, AgNP-, citrate-coated AuNP and TiO_2NP-exposed cells. These agglomerates mainly occurred at higher concentrations, with less and smaller sized agglomerates observed at lower concentrations.

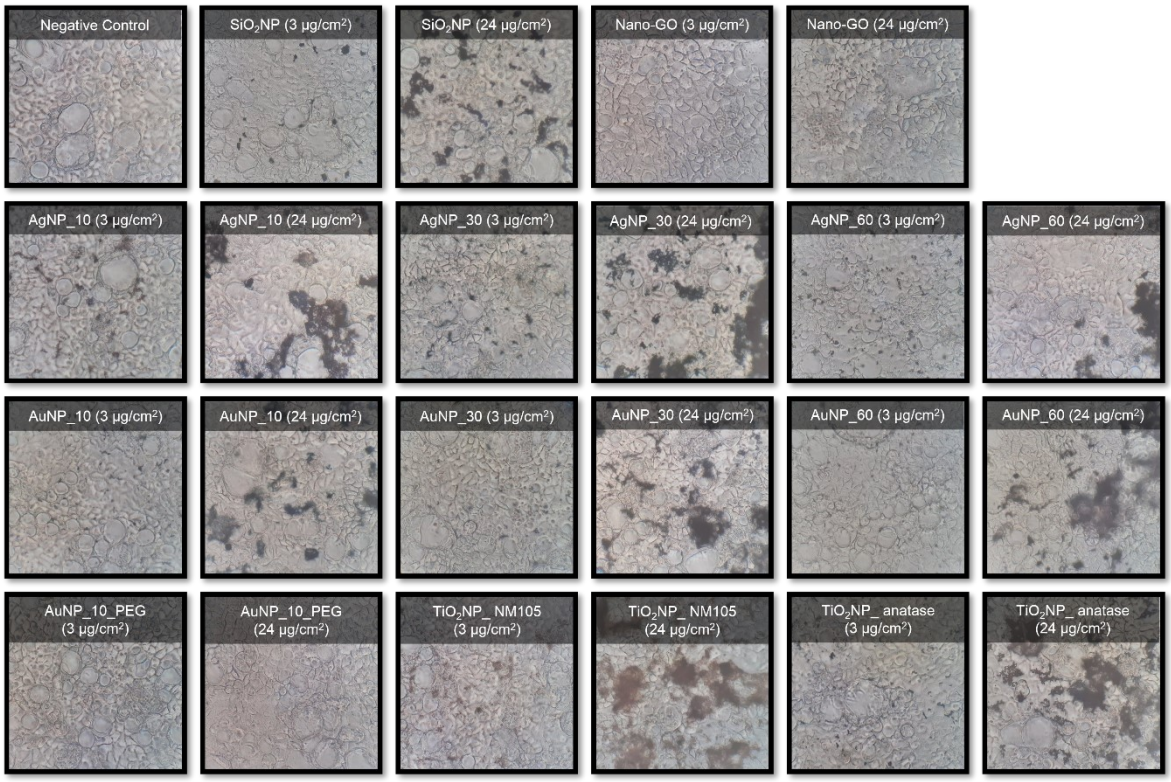

Figure 11. Representative optical micrographs of Caco-2 cells after 24 h in the presence or in the absence of the tested NP (400× magnification).

4.1.2. Cell metabolic activity and plasma membrane integrity

Overall, all tested AgNP reduced the metabolic activity of Caco-2 cells in a concentration- and size-dependent manner, as shown in **Figure 6A**. We found that the metabolic activity of cells exposed to the highest tested concentration (48 µg/cm^2) of

AgNP_10, AgNP_30 and AgNP_60 significantly decreased to 45.8 ± 7.3%, 52.6 ± 8.9% and 68.8 ± 15.5%, respectively, compared to the NC. Similar findings have been reported by Gillois *et al.* (2021) in Caco-2 cells exposed for 48 h to AgNP, where a concentration-dependent cell viability reduction was observed, as measured using the AlamarBlue assay.

Moreover, Caco-2 cells incubated with increasing concentrations of AgNP for 24 h did not exhibit significant changes in LDH release at any concentration compared to the controls (**Figure 6B**). According to Johnston *et al.* (2010), AgNP can pass through the GIT into the blood and thus distribute throughout the body. AgNP and Ag^+ are reported to induce toxicity by affecting toxicity pathways, with the potential to induce cellular responses involved in cell survival or death pathways (Gurunathan *et al.*, 2015). It has been hypothesized that AgNP-induced toxicity results from both Ag in the nanoparticulate form and released ions. However, De Matteis *et al.* (2015) showed that Ag^+ ions released into cell culture medium played an insignificant role in AgNP-induced toxicity. Herein, we found that AgNP-induced cytotoxicity by causing a decrease in metabolic activity in a concentration- and size-dependent manner, with smaller NP causing a more marked effect. On the other hand, LDH levels did not change compared to the NC.

Regarding AuNP, at 24 h after exposure to AuNP_10_PEG, no significant changes in cell viability of Caco-2 cells were found (**Figure 6C**). However, citrate-capped AuNP significantly affected Caco-2 cell metabolic activity at 24 h after exposure, in a concentration-dependent manner. Metabolic activity of cells exposed to 12, 24 and 48 μg/cm^2 of 10 nm AuNP, decreased to 75, 64 and 56%, respectively compared to NC. In addition, when cells were exposed to 6, 12, 24 and 48 μg/cm^2 of 30 nm AuNP, cell metabolic activity decreased to 87, 81, 70 and 58% compared to the NC, respectively. For cells exposed to 60 nm AuNP, the viability of cells exposed to the two highest concentrations tested (24 and 48 μg/cm^2) decreased, respectively, to 77 and 64%. Regarding LDH release, no significant changes compared to the NC after incubation with all tested AuNP were found, except in cells exposed to 6 μg/cm^2 of AuNP_10 and AuNP_10_PEG (**Figure 6D**).

PEG is one of the most used polymers to functionalize NP, as it can increase colloidal stability in a physiological environment, inhibit the nonspecific binding of other molecules on the surface of NP, improving their biocompatibility and providing conjugation sites for therapeutic agents and targeting ligands (Uz *et al.*, 2016). In this study, AuNP_10_PEG did not induce significant cytotoxic effects, either in terms of metabolic activity or LDH release which indicates that PEG is a protective coating that effectively prevents cytotoxic damage to cells.

Regarding the analysis of cell viability using the AlamarBlue assay for TiO_2NP (**Figure 6E**), a slight reduction in the metabolic activity of intestinal cells after 24 h was observed for rutile-anatase TiO_2NP_NM105, at the two highest tested concentrations (24

and 48 µg/cm^2), decreasing from 100.0 ± 3.0% to 80.3 ± 9.7% at the highest concentration tested. On the other hand, TiO_2NP_anatase did not induce any significant decrease in the metabolic activity of these cells. Moreover, both rutile-anatase and anatase TiO_2NP failed to affect plasma membrane integrity (**Figure 6F**) of Caco-2 cells after 24 h of exposure.

In the present study, we used two tests to determine the cytotoxic potential of the tested M-NP. The analysis of the metabolic activity changes indicated that all tested M-NP were cytotoxic to Caco-2 cells, except TiO_2NP_anatase and AuNP_10_PEG. Overall, the AlamarBlue assay revealed more sensitivity for detecting particle toxicity compared to the LDH assay, which can be explained by the fact that each assay evaluates different toxicity aspects: the metabolic activity of cells, an early toxicity effect, plasma membrane integrity, a late toxicity effect. Accordingly, our data highlight the importance of using more than one cytotoxicity assay for NM toxicity evaluation. Importantly, no significant interferences of the tested M-NP in these two assays have been detected, in the LDH release assay since no significant changes in the maximum release of LDH in the absence (PC) or in the presence of the highest M-NP tested concentration (PC+48 µg/cm^2) have been detected.

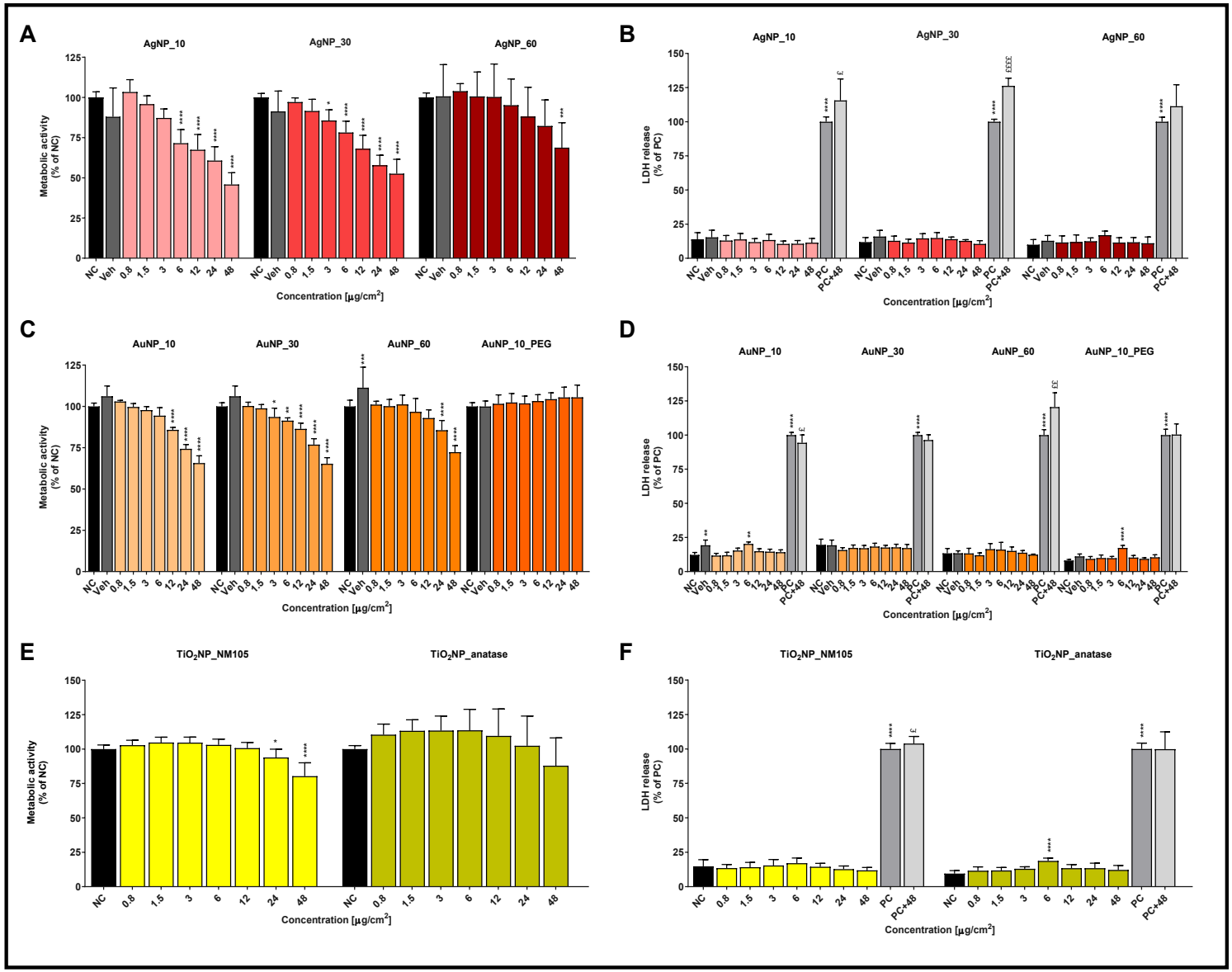

Figure 12. Metabolic activity and lactate dehydrogenase (LDH) release in Caco-2 cells after exposure for 24 h to metal-based nanoparticles (M-NP). Cells were exposed to various concentrations of differently sized (10, 30, 60 nm) **(A, B)** silver (AgNP), **(C, D)** gold (AuNP) and **(E, F)** rutile-anatase (TiO_2NP_NM105) and anatase (TiO_2NP_anatase) titanium nanoparticles. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC (negative control); £p < 0.05, ££p < 0.01 and ££££p < 0.0001 vs PC (positive control); Veh: Vehicle (16% H_2O in FBS-free DMEM).

For comparison with M-NP and to understand if the potential mechanisms by which they cause toxicity are similar, two other NM were tested: SiO_2NP and nano_GO. AlamarBlue assay showed that Caco-2 cells metabolic activity was inhibited to a greater extension by nano_GO than by SiO_2NP. After 24 h of incubation with different concentrations of SiO_2NP and nano_GO, Caco-2 cells showed a significant decrease in metabolic activity compared to the NC at the highest tested concentration (48 μg/cm^2) of SiO_2NP and at 24 and 48 μg/cm^2 of nano_GO (**Figure 7A**). Indeed, metabolic activity decreased to 80.6 ± 8.5% in the highest concentration of SiO_2NP, and to 51.7 ± 11.3% in the cells exposed to nano_GO.

Regarding plasma membrane integrity, LDH release in Caco-2 cells exposed to SiO_2NP for 24 h was marked increased at the two highest tested concentrations (24 and 48 μg/cm^2, corresponding to 80 and 160 μg/mL). Like in our study, Mortensen *et al.* (2020) also observed an increase in LDH release in Caco-2 cells exposed to 50 μg/mL of 15 nm SiO_2NP. On the other hand, nano_GO only increased LDH release at the concentration of 6 μg/cm^2 (20 μg/mL) (**Figure 7B**). Both SiO_2NP and nano_GO induced a decrease in metabolic activity from the concentration of 20 μg/mL. Thus, the effects on metabolic activity were more visible in intestinal cells exposed to nano_GO and only cells exposed to 6 μg/cm^2 SiO_2NP released levels of LDH statistically higher than the NC, indicating that this NM affect plasma membrane, while nano_GO act at the level of the metabolic capability of Caco-2 cells. In addition, both NM also did not interfere in LDH release measurements.

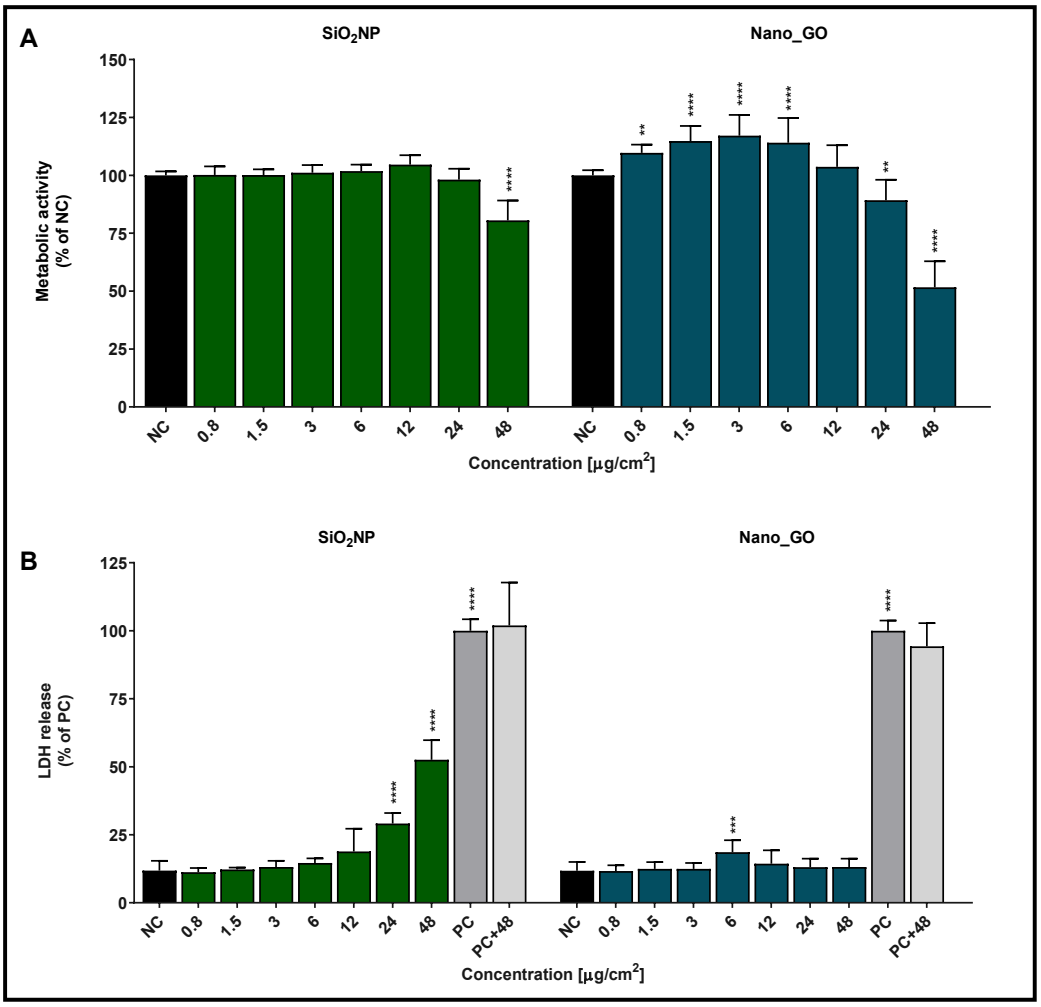

Figure 7. (A) Metabolic activity and **(B)** lactate dehydrogenase (LDH) release in Caco-2 cells after exposure for 24 h to various concentrations of non-metallic nanomaterials. Cells were exposed to silica nanoparticles (SiO_2NP) and nano-graphene oxide (nano_GO). Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC (negative control).

4.1.3. Oxidative stress: ROS levels

Oxidative stress in response to the tested NM was determined by measuring ROS levels in both cell models following 24 h of exposure. ROS are chemical species produced as by-products of cellular oxygen metabolism, which occurs through mitochondrial respiration in eukaryotic cells and are general mediators of cytotoxicity induced by various features of NP (Nel *et al.*, 2006). Therefore, we were interested in evaluating whether the chosen NM could increase intracellular ROS in Caco-2 cells.

In **Figure 8A** are represented ROS levels in Caco-2 cells after a 24 h incubation period with AgNP. Caco-2 cells show a significant increase in ROS levels in cells exposed to 0.8-12 µg/cm^2 (2.5-40 µg/mL) of 10 nm AgNP, 3-24 µg/cm^2 (10-80 µg/mL) of 30 nm AgNP and 6-24 µg/cm^2 (20-80 µg/mL) of 60 nm AgNP compared to the NC. On the contrary, Song

et al. (2014) observed that 90 nm AgNP did not significantly induce ROS in the concentration range of 0-200 µg/mL in Caco-2 cells at 24 h after exposure. This may be explained by differences in the experimental protocol between the two studies, because, for example, here the cells were incubated with the probe for 1 h, whereas in the study by Song *et al.* (2014), the incubation was only for 30 min. In addition, the AgNP used in that study had a size of 90 nm, that is, they were larger than any of the AgNP tested here. For instance, here we found that ROS levels increased less with the increasing size of the AgNP.

Regarding the effect of AuNP in the generation of ROS from Caco-2, only a significant increase was observed for a lower concentration (3 and 6 µg/cm^2) of AuNP_10_PEG, compared to the corresponding control (**Figure 8B**).

Figure 8C shows the ROS levels after exposure to TiO_2NP. None of the tested TiO_2NP induced significant changes in ROS levels of Caco-2 cells comparing with NC cells. Our results are in accordance with the published literature which shows that the general toxicity of TiO_2 is low, even at high concentrations (Shi *et al.*, 2013).

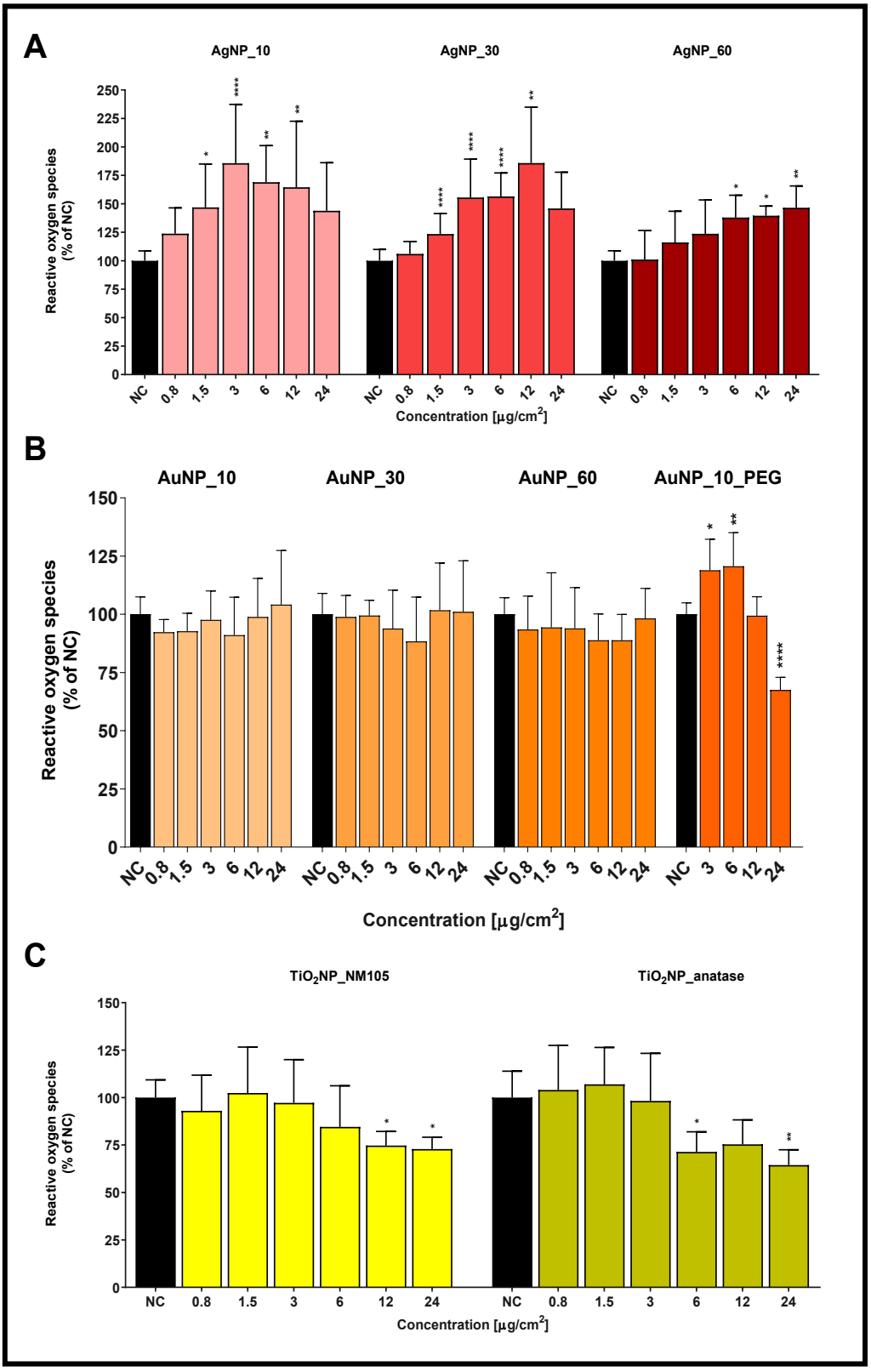

Figure 8. Reactive oxygen species (ROS) levels in Caco-2 cells after exposure for 24 h to various concentrations of metal nanoparticles (M-NP). Cells were exposed to differently sized (10, 30, 60 nm) **(A)** silver (AgNP), **(B)** gold (AuNP) and **(C)** rutile-anatase (TiO_2NP_NM105) and anatase (TiO_2NP_anatase) titanium nanoparticles. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ vs NC (negative control).

Regarding non-metallic NM, the results showed that there is no significant effect on ROS levels at any SiO_2NP or nano_GO tested concentrations (**Figure 9**). Only AgNP and AuNP induced an increase in the levels of ROS produced by Caco-2 cells. Regarding AgNP, this increase was visible in all sizes tested, highlighting the toxicity of these NP to intestinal cells. Interestingly, in AuNP, there was only an effect at the two lowest tested concentrations of AuNP_10_PEG. Therefore, although these AuNP do not cause a decrease in metabolic capability or significantly impact plasma membrane integrity, the same is not true for the oxidative stress induced by the production of ROS. Some studies have indicated that carbon nanoparticles (CNP) can generate ROS in mammalian cells (Pulskamp *et al.*, 2007; Sanchez *et al.*, 2012), however, in this study, the carbon-based NM used, nano_GO, did not induce the generation of ROS at any tested concentration. This may be related to the sensitivity of cell line that is used in each study, as different cells may have different responses to NM.

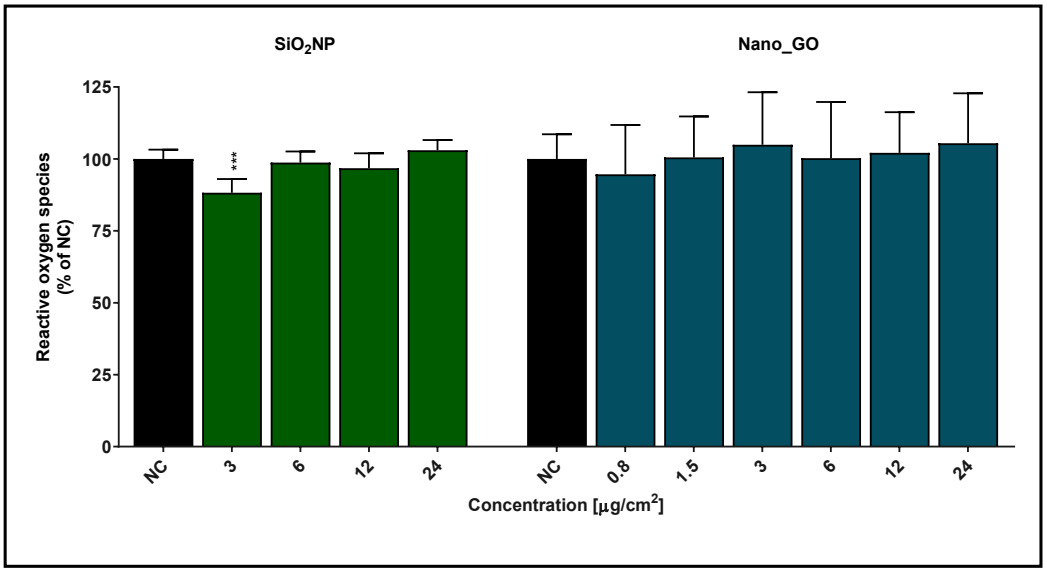

Figure 9. Reactive oxygen species (ROS) levels in Caco-2 cells after exposure for 24 h to various concentrations of non-metallic nanomaterials. Cells were exposed to silica nanoparticles (SiO_2NP) and nano-graphene oxide (nano_GO). Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. ***$p < 0.001$ vs NC (negative control).

4.1.4. Determination of intracellular ATP levels

The results obtained for the ATP assay for Caco-2 cells are shown in **Table 2**. Exposure to all tested AgNP induced a significant increase in the intracellular levels of ATP, though more evident in the smaller sized AgNP_10, that follow a clear concentration-dependent trend.

Regarding AuNP, a significant effect on the amount of intracellular ATP at concentrations of 3, 12 and 24 μg/cm^2 of AuNP_10, 12 μg/cm^2 of AuNP_30 and at the highest concentration of AuNP_60 and AuNP_10_PEG was observed.

The amount of ATP generated by Caco-2 cells increased significantly compared to the NC when they were exposed to 12 and 24 μg/cm^2 of TiO_2NP_NM105. For TiO_2NP_anatase, a concentration-dependent increase in intestinal epithelial cells ATP levels was detected. Notably, TiO_2NP exhibited toxicological effects on Caco-2 cells, increasing the amount of ATP generated. The same was not observed by Fisichella *et al.* (2012), since in their study the presence of TiO_2NP [coated with an aluminium hydroxide $Al(OH)_3$ layer and an outer layer of polydimethylsiloxane (PDMS)] did not induce a toxic effect on Caco-2 cells after 24 h of exposure, using the ATP assay. These differences between the two studies may lie on the type of tested NP, since in the study, coated TiO_2NP were used, while in our study TiO_2NP do not have any surface coating.

SiO_2NP significantly increased ATP levels in Caco-2 cells in a concentration-dependent manner. On the other hand, nano_GO provoked a marked increase of intracellular ATP at concentrations of 3, 6 and 12 μg/cm^2, but no differences compared to NC were detected at the highest tested concentration.

Apoptosis is a form of cell death that requires energy. Zamaraeva *et al.* (2005) suggested that elevation of the cytosolic ATP level is a requirement for the process of cell death by apoptosis. Consequently, when measuring ATP, one must consider that elevation in intracellular ATP levels might be related with its increased production for programmed cell death activation. Nevertheless, increased levels of ATP in response to NM might also be related with reduced ATP utilization.

Table 2. Effect of the different tested nanomaterials (NM) on ATP levels by Caco-2 cells. Cells were exposed to different concentrations of the tested NM for 24 h and to 10 mM NaF (positive control) for 3h. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, ***p < 0.001 and ****p < 0.0001 vs NC (negative control).

Experimental Group	Concentration	ATP content (% of NC)
Negative Control (NC)		100.0 ± 13.6
NaF (Positive control)	**10 mM**	**9.9 ± 1.1 ******
AgNP_10 nm	**3 µg/cm²**	**120.0 ± 10.2 ***
	6 µg/cm²	**133.2 ± 10.8 *****
	12 µg/cm²	**169.0 ± 15.0 ******
	24 µg/cm²	**178.2 ± 14.8 ******
AgNP_30 nm	**3 µg/cm²**	**129.8 ± 14.9 ****
	6 µg/cm²	**144.1 ± 20.1 ******
	12 µg/cm²	**139.7 ± 14.9 ******
	24 µg/cm²	**156.6 ± 15.7 ******
AgNP_60 nm	3 µg/cm²	116.9 ± 7.4
	6 µg/cm²	**123.8 ± 14.0 ***
	12 µg/cm²	**137.5 ± 18.1 ******
	24 µg/cm²	**136.9 ± 16.6 ******
AuNP_10 nm	**3 µg/cm²**	**129.2 ± 18.7 ***
	6 µg/cm²	120.8 ± 5.9
	12 µg/cm²	**133.7 ± 19.0 ****
	24 µg/cm²	**129.1 ± 29.2 ***
AuNP_30 nm	3 µg/cm²	114.3 ± 26.8
	6 µg/cm²	117.4 ± 20.6
	12 µg/cm²	**176.9 ± 88.8 ****
	24 µg/cm²	146.2 ± 52.0
AuNP_60 nm	3 µg/cm²	98.0 ± 7.4
	6 µg/cm²	110.2 ± 12.2
	12 µg/cm²	115.9 ± 7.6
	24 µg/cm²	**117.9 ± 14.0 ***
AuNP_10_PEG	3 µg/cm²	122.6 ± 48.7
	6 µg/cm²	134.5 ± 39.4
	12 µg/cm²	149.6 ± 50.6
	24 µg/cm²	**177.0 ± 86.6 ***
TiO_2NP_NM105	**3 µg/cm²**	**127.4 ± 22.1 ***
	6 µg/cm²	119.5 ± 14.4
	12 µg/cm²	**142.3 ± 19.5 *****
	24 µg/cm²	**129.9 ± 15.7 ****
TiO_2NP_anatase	3 µg/cm²	118.8 ± 8.7
	6 µg/cm²	**126.7 ± 11.3 ***
	12 µg/cm²	**143.8 ± 18.1 ******
	24 µg/cm²	**152.7 ± 26.9 ******
SiO_2NP	**3 µg/cm²**	**131.4 ± 8.5 ***
	6 µg/cm²	**172.3 ± 4.0 ******
	12 µg/cm²	**155.6 ± 15.4 *****
	24 µg/cm²	**232.4 ± 12.6 ******
Nano_GO	**3 µg/cm²**	**155.7 ± 14.6 *****
	6 µg/cm²	**141.7 ± 20.7 ****
	12 µg/cm²	**163.0 ± 16.6 *****
	24 µg/cm²	117.0 ± 10.1

4.2. Placental barrier (BeWo b30 cells)

4.2.1. Monitoring of cell morphological changes by optical microscopy

Figure 10 shows optical microscopy images of trophoblastic BeWo b30 cells after exposure for 24 h to the tested NM. Like Caco-2 cells, no evident changes in cellular morphology have been detected following exposure to the M-NP and non-metallic NM, while NM clusters were also visible, mainly at higher concentrations.

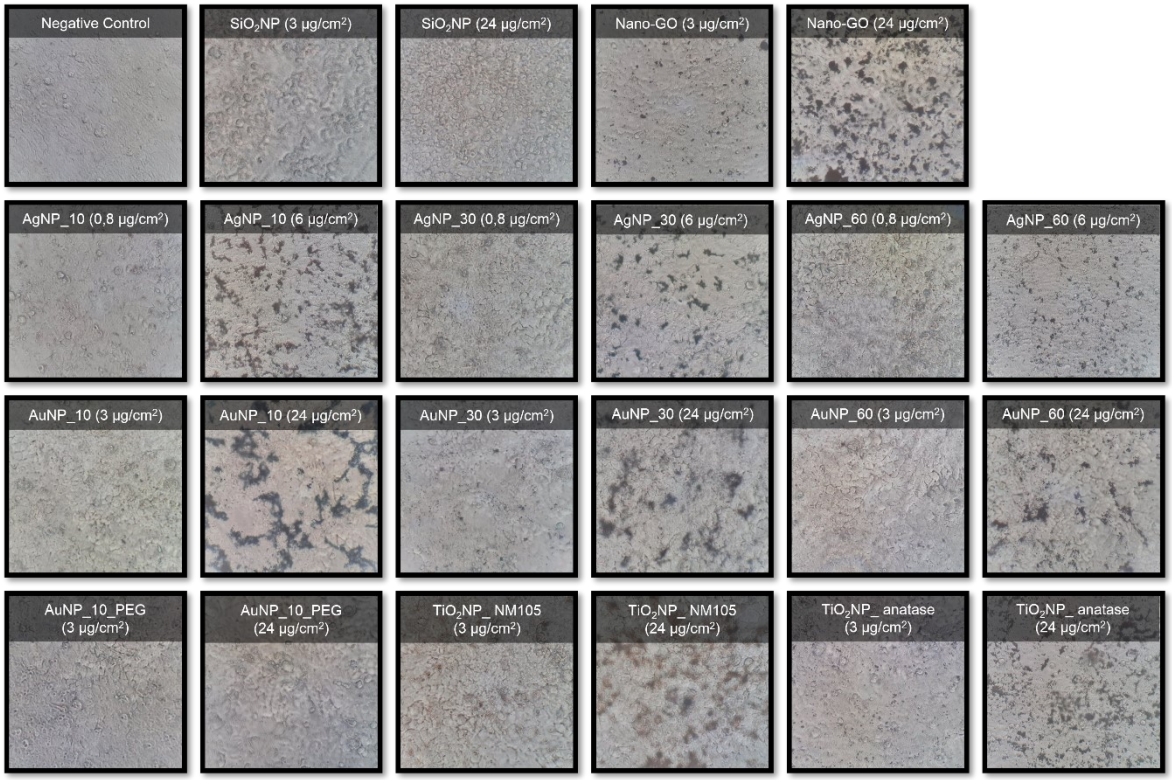

Figure 10. Representative optical micrographs of BeWo b30 cells after 24 h in the presence or in the absence of the tested NP (400× magnification).

4.2.2. Cell metabolic activity and plasma membrane integrity

In **Figure 11A** are represented the results concerning cellular metabolic activity in BeWo b30 cells after exposure to AgNP, as measured by the AlamarBlue assay. A size- and concentration-dependent decrease in the metabolic activity have been observed. For the highest tested AuNP concentration, reductions of 22.4 ± 3.4%, 46.6 ± 4.2% and 50.0 ± 6.7% relative to the NC were obtained for AuNP_10, AuNP_30 and AuNP_60, respectively. Decrease in cellular metabolic activity was accompanied by changes in plasma membrane integrity as shown in **Figure 11B**. Thus, cells exposed to AgNP had their metabolic activity

reduced in a concentration- and size-dependent manner, with the highest concentrations and the smallest sized being the most toxic. In this case, the same was verified in the assay where the release of LDH was determined, in which the amount of extracellular LDH increased in cells exposed to the highest concentrations of AgNP, this increasing being more accentuated for the smaller AgNP.

The effect of AuNP on the metabolic activity of trophoblastic cells was also measured (**Figure 11C**). All variants except for AuNP_10_PEG, significantly reduced the metabolic activity of BeWo cells. For the highest tested concentration, metabolic activity decreased to 64.3 ± 2.2%, 65.6 ± 5.0% and 73.9 ± 5.9% in cells exposed to AuNP_10, AuNP_30 and AuNP_60, respectively. In contrast, incubation with AuNP did not significantly change membrane integrity at the concentrations and time point tested (**Figure 11D**). Citrate-capped AuNP reduced the metabolic activity of placental cells, a decrease that was more evident in AuNP_10 exposed cells. Once again, PEG-capping conferred a protective effect to AuNP, attenuating their effects upon metabolic activity of the cells exposed to them. For LDH release, only an intermediate concentration of AuNP_10_PEG (6 μg/cm^2 that correspond to 20 μg/mL) induced a significant increase, and under all other conditions no effects were observed compared to the NC. Our results contrast with those reported by Aengenheister *et al.* (2018) where no differences in the viability of BeWo b30 cells exposed for up to 48 h to 4 nm AuNP concentrations ranging from 0 to 50 μg/mL were observed. Again, this may be due to differences in the experimental conditions, in this case different assays to assess cell viability (AlamarBlue vs MTS assay) but specially to differences in AuNP physicochemical characteristics used in each study.

Cell viability was also altered by exposure to TiO_2NP_NM105 and TiO_2NP_anatase. Compared to the NC, cells exposed to the highest concentration of rutile-anatase TiO_2NP_NM105 showed, a decrease in metabolic capacity to 79.2 ± 3.2%, while in TiO_2NP_anatase exposed cells a reduction to 78.4 ± 4.0% was observed (**Figure 11E**). A slight but significant increase in LDH release was observed in cells exposed to high concentrations of both tested TiO_2NP (**Figure 11F**). Thus, our data seem to indicate that the crystallinity of TiO_2NP do not play an important role in terms of the toxicity outcome in BeWo b30 cells.

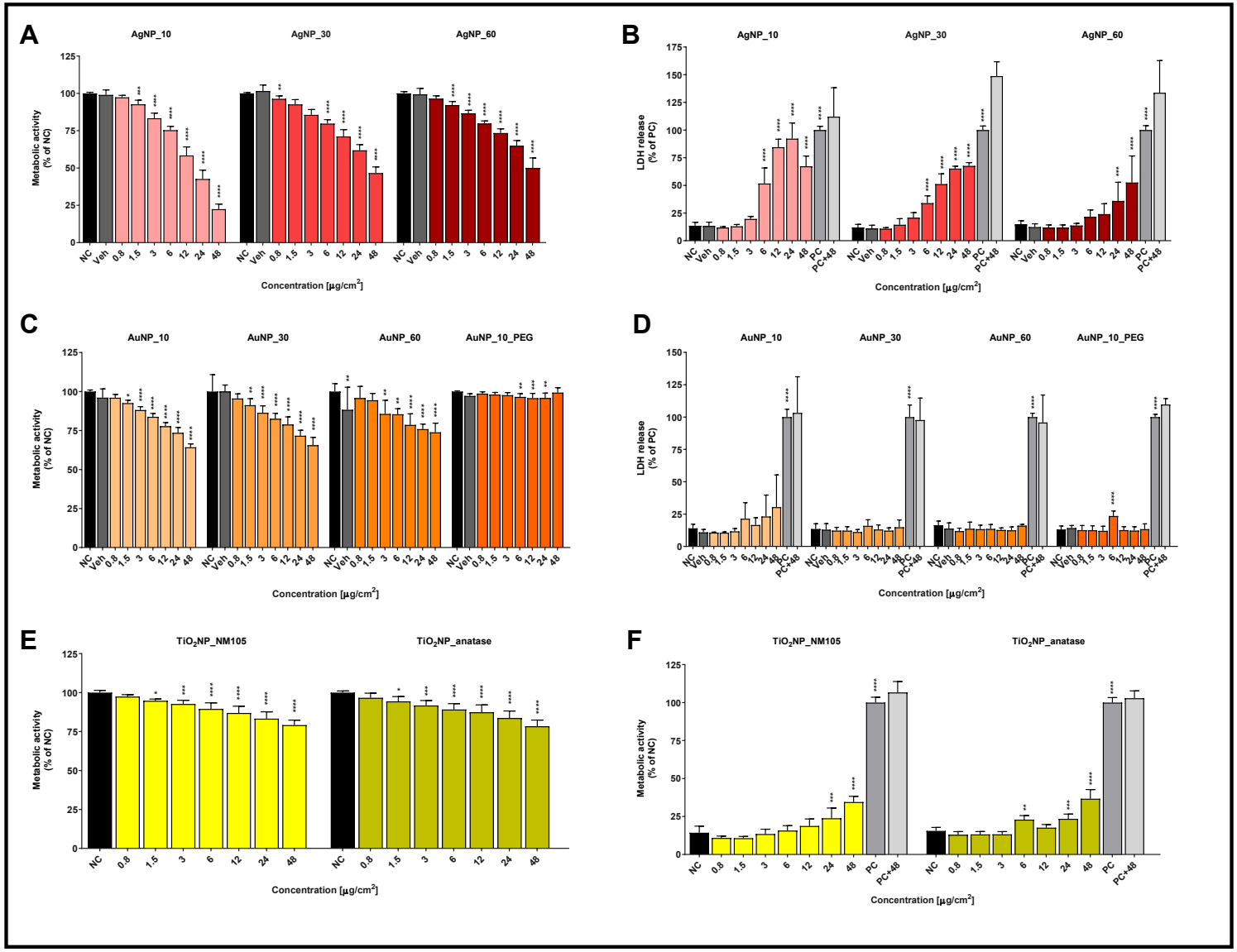

Figure 11. Metabolic activity and lactate dehydrogenase (LDH) release in BeWo b30 cells after exposure for 24 h to metal-based nanoparticles (M-NP). Cells were exposed to various concentrations of differently sized (10, 30, 60 nm) **(A, B)** silver (AgNP), **(C, D)** gold (AuNP) and **(E, F)** rutile-anatase (TiO_2NP_NM105) and anatase (TiO_2NP_anatase) titanium nanoparticles. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC (negative control); £££p < 0.001 and ££££p < 0.0001 vs PC (positive control); Veh: Vehicle (16% H_2O in FBS-free DMEM).

Regarding non-metallic NM, SiO_2NP significantly inhibited BeWo b30 metabolic activity only at the highest tested concentration (**Figure 12A**). Likewise, LDH assay showed that SiO_2NP induced a significant release of the enzyme at highest concentrations (**Figure 12B**). On the other hand, nano_GO significantly inhibited BeWo b30 viability, in a concentration-dependent manner (**Figure 12A**). However, no relevant changes in LDH release were detected in nano_GO-exposed cells (**Figure 12B**).

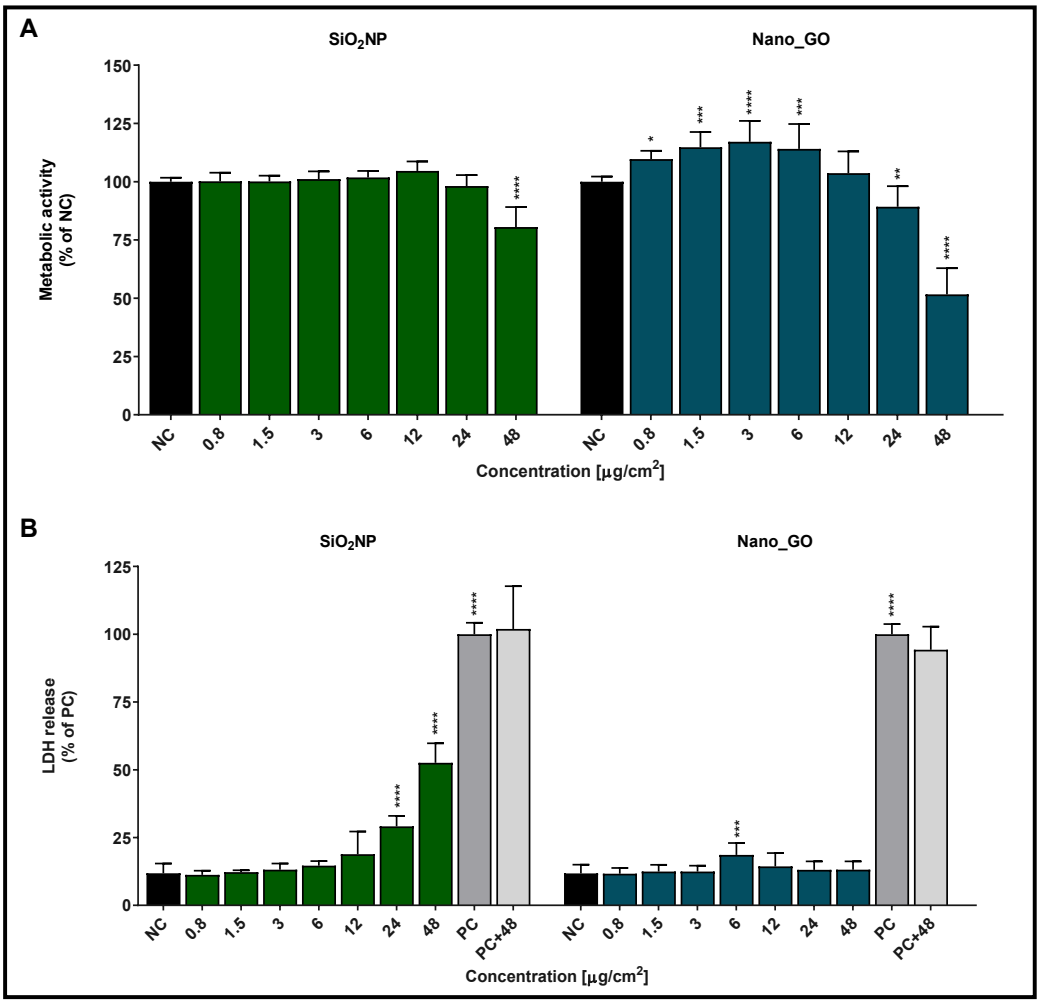

Figure 12. (A) Metabolic activity and **(B)** lactate dehydrogenase (LDH) release in BeWo b30 cells after exposure for 24 h to various concentrations of non-metallic nanomaterials. Cells were exposed to silica nanoparticles (SiO_2NP) and nano-graphene oxide (nano_GO). Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC (negative control).

4.2.3. Oxidative stress: ROS levels

All tested AgNP induced an increase in ROS levels in BeWo b30 cells after 24 h of exposure. AgNP_10 and AgNP_30 exposed cells exhibited higher levels compared to AgNP_60 (**Figure 13 A**). On the other hand, citrate capped AuNP did not increase ROS levels in trophoblastic cells, however AuNP_10_PEG at 3 and 6 μg/cm^2 significantly increased ROS levels compared to control (**Figure 13B**). TiO_2NP did not change ROS levels compared to the NC, regardless their crystallinity and concentration (**Figure 13C**).

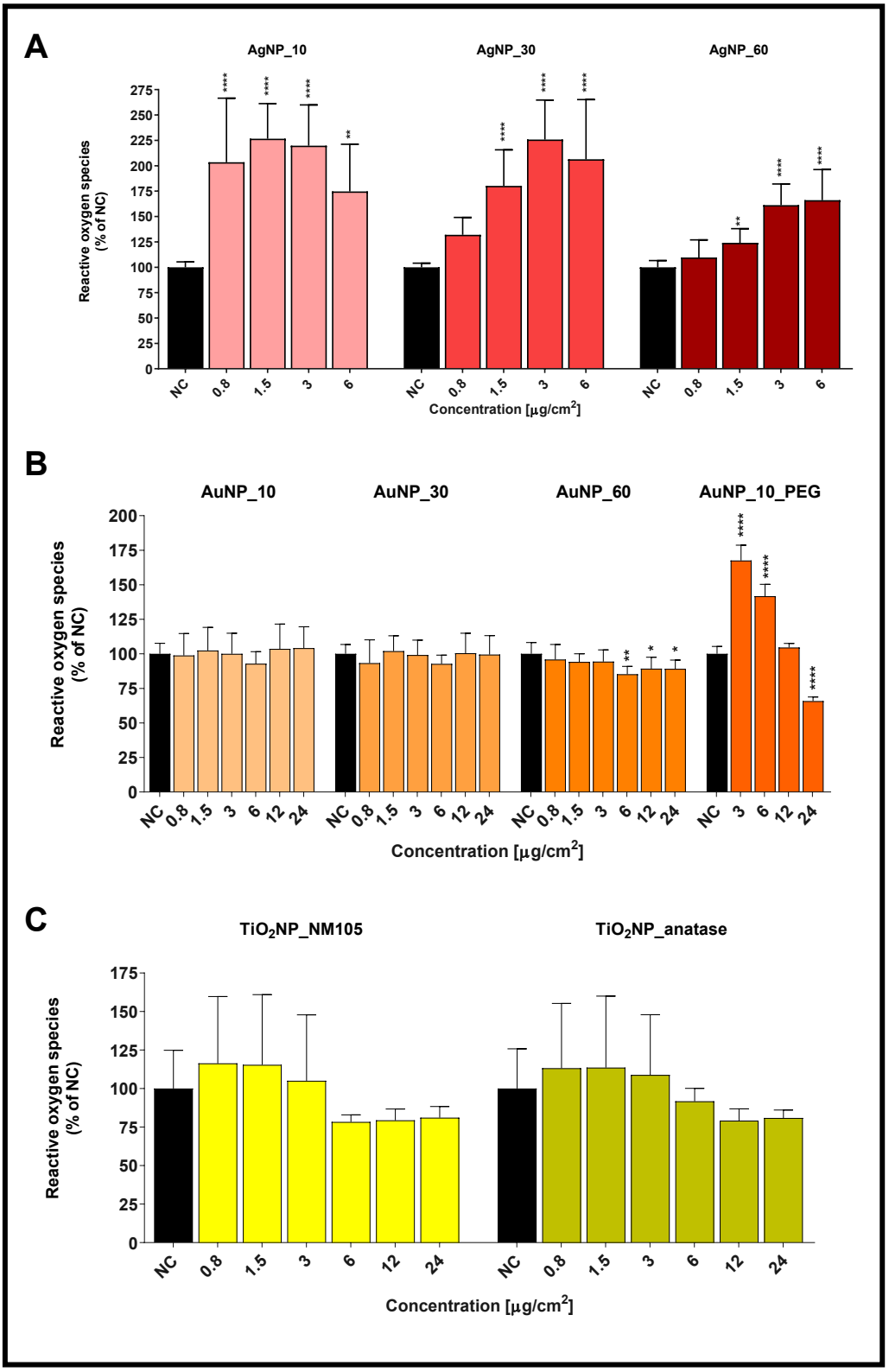

Figure 13. Reactive oxygen species (ROS) levels in BeWo b30 cells after exposure for 24 h to various concentrations of metal nanoparticles (M-NP). Cells were exposed to differently sized (10, 30, 60 nm) **(A)** silver (AgNP), **(B)** gold (AuNP) and **(C)** rutile-anatase (TiO_2NP_NM105) and anatase (TiO_2NP_anatase) titanium nanoparticles. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, and ****p < 0.0001 vs NC (negative control).

BeWo b30 cells exposed to SiO_2NP, exhibited a relatively modest but significant effect on ROS levels (**Figure 14**). At low concentration (6 µg/cm^2), nano_GO slightly increased ROS levels, however high concentrations induced a marked effect on ROS levels in trophoblastic cells up to 235% of the NC.

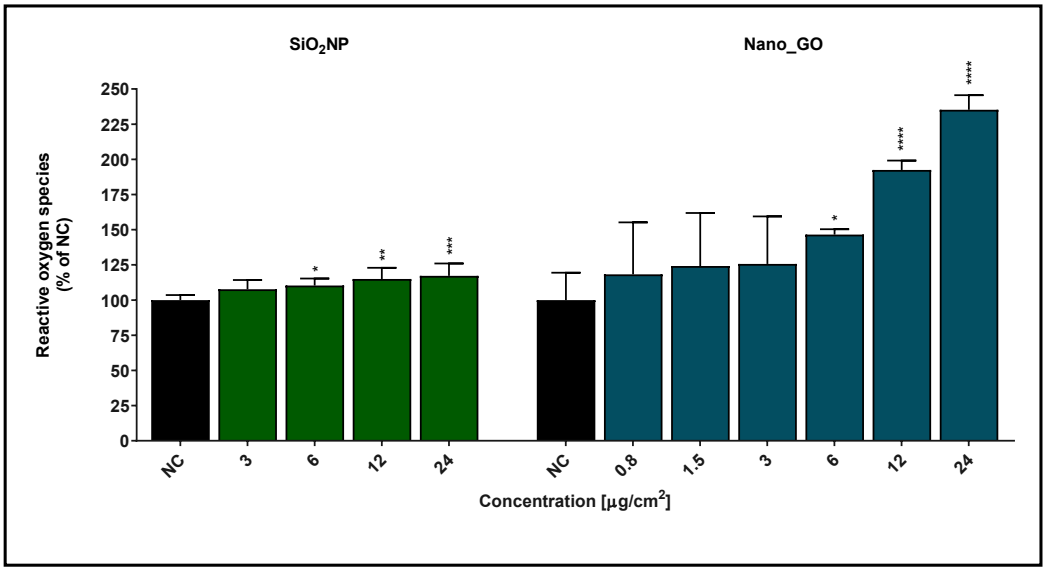

Figure 14. Reactive oxygen species (ROS) levels in BeWo b30 cells after exposure for 24 h to various concentrations of non-metallic nanomaterials. Cells were exposed to silica nanoparticles (SiO_2NP) and nano-graphene oxide (nano_GO). Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs NC (negative control).

4.2.4. Determination of intracellular ATP levels

The results obtained for the ATP assay in BeWo b30 cells are shown in **Table 3**.

AgNP-treated cells exhibited significantly increased intracellular ATP levels compared to control cells, but no clear concentration-dependence was observed. Regarding AuNP, some significant changes in ATP levels have been observed. While AuNP_10 did not induce changes in ATP levels, AuNP_30 slightly increased ATP levels but only at 12 µg/cm^2, whereas AuNP_60-exposed cells exhibited increased levels at both 6 and 12 µg/cm^2. Interestingly, increased level of intracellular ATP has been detected in cells exposed to all tested AuNP_10_PEG concentrations.

TiO_2NP_NM105 did not affect trophoblast cell ATP levels up to 6 µg/cm^2 but caused a significant increase at 12 and 24 µg/cm^2. In contrast, TiO_2NP_anatase induced an

increase in ATP levels at concentrations of 3 and 6 μg/cm^2, but not at the highest tested concentrations (12 and 24 μg/cm^2).

In the placental cell model, SiO_2NP induced a slight but significant increase in ATP levels at the two highest tested concentrations (12 and 24μg/cm^2). On the other hand, BeWo b30 cells exposed to nano_GO showed significantly increased ATP levels at lower tested concentration (3 μg/cm^2), whereas a decrease in intracellular ATP was found in cells exposed to the highest tested concentration (24 μg/cm^2).

As discussed above for Caco-2 cells, changes in ATP levels are likely to be explained by changes in ATP production and/or utilization in response to NM exposure.

Table 3. Effect of the different tested nanomaterials (NM) on ATP levels by BeWo b30 cells. Cells were exposed to different concentrations of the tested NM for 24 h and to 10 mM NaF (positive control) for 3h. Data is expressed as mean ± SD. Data was analysed by the one-way analysis of variance (ANOVA) test followed by the Dunnett's post hoc test for multiple comparisons. *p < 0.05, ***p < 0.001 and ****p < 0.0001 vs NC (negative control).

Experimental Group	Concentration	ATP content (% of NC)
Negative Control (NC)		100.0 ± 13.3
NaF (Positive control)	**10 mM**	**22.5 ± 5.2 ******
AgNP_10 nm	**0.8 µg/cm²**	**161.5 ± 12.7 ******
	1.5 µg/cm²	131.6 ± 29.3
	3 µg/cm²	**188.1 ± 34.0 ******
	6 µg/cm²	**148.9 ± 21.4 ****
AgNP_30 nm	**0.8 µg/cm²**	**144.1 ± 38.8 ***
	1.5 µg/cm²	**159.0 ± 31.9 *****
	3 µg/cm²	**169.7 ± 20.6 *****
	6 µg/cm²	**177.5 ± 23.2 ******
AgNP_60 nm	**0.8 µg/cm²**	**139.4 ± 17.8 ***
	1.5 µg/cm²	**145.8 ± 11.7 ****
	3 µg/cm²	**167.8 ± 36.5 ******
	6 µg/cm²	**145.4 ± 23.4 ****
AuNP_10 nm	3 µg/cm²	107.6 ± 20.4
	6 µg/cm²	109.7 ± 13.4
	12 µg/cm²	107.8 ± 14.1
	24 µg/cm²	106.4 ± 14.0
AuNP_30 nm	3 µg/cm²	92.4 ± 10.9
	6 µg/cm²	107.5 ± 30.9
	12 µg/cm²	**121.4 ± 7.2 *****
	24 µg/cm²	147.6 ± 24.4
AuNP_60 nm	3 µg/cm²	121.1 ± 8.0
	6 µg/cm²	**134.3 ± 19.1 ****
	12 µg/cm²	**138.3 ± 13.4 ****
	24 µg/cm²	126.2 ± 29.6
AuNP_10_PEG	**3 µg/cm²**	**129.2 ± 24.0 ***
	6 µg/cm²	**143.7 ± 6.2 ****
	12 µg/cm²	**142.9 ± 38.3 ****
	24 µg/cm²	**163.5 ± 10.6 ******
TiO_2NP_NM105	3 µg/cm²	121.0 ± 23.4
	6 µg/cm²	140.0 ± 14.8
	12 µg/cm²	**231.4 ± 25.4 ******
	24 µg/cm²	**213.9 ± 90.3 *****
TiO_2NP_anatase	**3 µg/cm²**	**171.9 ± 86.4 ***
	6 µg/cm²	**242.2 ± 59.5 ******
	12 µg/cm²	144.6 ± 39.3
	24 µg/cm²	117.6 ± 15.3
SiO_2NP	3 µg/cm²	123.8 ± 19.8
	6 µg/cm²	109.2 ± 17.3
	12 µg/cm²	**146.2 ± 20.4 ***
	24 µg/cm²	**153.9 ± 29.7 ***
Nano_GO	**3 µg/cm²**	**144.1 ± 24.1 ***
	6 µg/cm²	129.0 ± 20.0
	12 µg/cm²	84.1 ± 7.6
	24 µg/cm²	**66.2 ± 8.0 ***

4.3. Intestinal versus placental barrier

Our *in vitro* cytotoxicity data for AgNP revealed that these particles induce cytotoxicity in both cell lines in a concentration-dependent manner, although BeWo b30 cells exhibit greater sensitivity to these NP compared to Caco-2 cells. These findings agree with previous studies showing that exposure to AgNP can reduce cell viability and significantly increase LDH release and ROS production in a concentration-dependent manner (AshaRani *et al.*, 2009; Liu *et al.*, 2015). In this context, Park *et al.* (2011) also investigated the effects of differently sized AgNP (20, 80 and 113 nm) using *in vitro* assays for cytotoxicity and concluded that 20 nm AgNP were more toxic than the larger AgNP. Several studies have found that AgNP can potentially elevate ROS levels in a variety of cells, evidencing a molecular mechanism of AgNP-mediated cytotoxicity (Hsin *et al.*, 2008; Mei *et al.*, 2012; Rahman *et al.*, 2009). The results of the present study and those of previous studies provide therefore evidence of a link between AgNP-mediated ROS production and cytotoxicity.

One of the major contributors behind AgNP-induced toxicity proposed is their ability to elevate ROS levels, leading to oxidative stress. It is believed that this mechanism is also important for the toxicity of other classes of manufactured NP.

The reduction in cell viability as well as the increase in plasma membrane damage and ROS production were more pronounced for AgNP-exposed cells compared to the other M-NP, mainly in the BeWo b30 cell line. After 24 h of exposure to 48 μg/cm^2 of AgNP, the viability of BeWo b30 cells was less than 23% compared to the 46% observed in Caco-2 cells. Clearly, our study showed that different cell types react differently to NM exposure. One reason for the different responses in intestinal and placental cells may be the robustness in tissue types and mucus layer, which has a protective function and is present at the top of intestinal cultures but absent in placental trophoblast cultures (Drasler *et al.*, 2017; Wright and Kelly, 2017). Like Bar-Ilan *et al.* (2009) demonstrated in zebrafish embryos, also here we could verify that AgNP and AuNP of similar size exhibited significantly different toxic effects, especially in ROS and ATP assays, suggesting that chemical composition is an important driver of NM toxicity. Furthermore, and as reviewed by Sani *et al.* (2021), Aueviriyavit *et al.* (2014) observed that AgNP but not AuNP induced acute cytotoxicity through the pathway related to oxidative stress in Caco-2 cells, as found here in the ROS assay, for both the intestinal and placental cell lines.

Acute exposure to TiO_2 particles caused some degree of cytotoxicity and increased ATP levels, but not intracellular ROS. Therefore, TiO_2NP cause only minimal toxicological impact on intestinal and placental models *in vitro*, with the impact being more prominent on trophoblastic cells.

Various parameters, such as chemical composition, size and surface coating have been shown to influence the interaction of particles with cells. The effect of TiO_2NP crystalline structure on toxicity was less pronounced compared to the differences observed in AgNP and AuNP with different sizes. In our studies with AgNP and AuNP of 10, 30 and 60 nm, differences were observed in terms of toxicity pattern, while with TiO_2NP_NM105 and TiO_2NP_anatase, very few differences were observed. The results obtained suggest that TiO_2NP induce less cytotoxicity than AuNP, with AgNP being the most cytotoxic NP. AgNP showed clear size-dependent toxicity in both cytotoxicity assays. The size of the NP is extraordinarily relevant to its cytotoxicity. However, the underlying mechanism for the size-dependent effects of NP remains unclear. It has been suggested that smaller AgNP more readily enter cells by diffusion across the cell membrane or by endocytosis. Subsequently, many internalized AgNP induce more cytotoxicity, producing ROS and, finally, inhibition of cell proliferation (McShan *et al.*, 2014).

5. Conclusions and Future Perspectives

5. Conclusions and Future Perspectives

The increasing use of NM in consumer, industrial and biomedical products has generated a global concern regarding its toxicity and impact on biological systems. Our study presents a mechanistic toxicological evaluation of two classes of NM of economic interest: M-NP and non-metallic NM. Two different epithelial cell models were used in the present study to elucidate on the potential toxicity of M-NP and non-metallic NM on biological barriers: human intestinal Caco-2 and trophoblastic BeWo b30 cells.

Our findings suggest that M-NP can be ranked for toxicity as AgNP > AuNP > TiO_2NP. Despite the toxicity profile of the tested M-NP being similar in both models of human barrier, placental cells showed a greater sensitivity than intestinal cells. Thus, our study highlights that it is important to evaluate each cell type individually since the same material can induce different effects in different models. Furthermore, this study also suggests that physicochemical properties are important determinants of NM toxicity, in particular chemical composition, size, and surface coating. The influence of size on M-NP toxicity was more evident for AgNP than for AuNP, with the smallest particles being more cytotoxic. Regarding the surface coating, PEG capping acted as an effective protection against AuNP-induced toxicity, preventing effects that are visible in cells exposed to the tested citrate-capped AuNP. The effects were also more visible in cells exposed to the highest tested concentrations of M-NP.

The production of ROS was evaluated, and it was determined that AgNP and AuNP_10_PEG increased intracellular ROS levels in both cell lines. Based on these results, it can be concluded that oxidative stress is a possible mechanism underlying the cytotoxicity induced by these M-NP in Caco-2 and BeWo b30 cells.

The *in vitro* systems used in this study were limited to individual cell types and therefore were not able to replicate the intricate interactions that occur *in vivo*. However, information obtained from *in vitro* studies can be useful to predict new targets for defining better strategies for *in vivo* evaluations. Further studies are underway to investigate the genotoxicity of the NM used in this study on intestinal and trophoblastic epithelial cells. Comet assay evaluation is being performed to detect primary and oxidative DNA damage in cells exposed to these NM. In the future, it is also intended to perform an analysis of the samples using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique to make an absolute quantification of the cellular uptake of M-NP. Additional research needs to be conducted in this field to achieve a deeper understanding of the toxicity of M-NP in human barriers, namely at the intestinal and placental levels, to unravel the mechanisms of action and the properties responsible for M-NP-mediated toxicity, and thus support

regulatory decisions that protect workers and consumers and ultimately assist in the development of safer NM manufacturing processes.

6. References

6. References

Abudayyak, M., Öztaş, E., Arici, M. and Özhan, G. (2017). Investigation of the toxicity of bismuth oxide nanoparticles in various cell lines. *Chemosphere, 169*, 117-123.

Aengenheister, L., Dietrich, D., Sadeghpour, A., Manser, P., Diener, L., Wichser, A., Karst, U., Wick, P. and Buerki-Thurnherr, T. (2018). Gold nanoparticle distribution in advanced *in vitro* and *ex vivo* human placental barrier models. *Journal of Nanobiotechnology, 16*(1), 79.

Aengenheister, L., Dugershaw, B. B., Manser, P., Wichser, A., Schoenenberger, R., Wick, P., Hesler, M., Kohl, Y., Straskraba, S., Suter, M. J. F. and Buerki-Thurnherr, T. (2019). Investigating the accumulation and translocation of titanium dioxide nanoparticles with different surface modifications in static and dynamic human placental transfer models. *European Journal of Pharmaceutics and Biopharmaceutics, 142*, 488-497.

Ahamed, M., Karns, M., Goodson, M., Rowe, J., Hussain, S. M., Schlager, J. J. and Hong, Y. (2008). DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells. *Toxicology and Applied Pharmacology, 233*(3), 404-410.

Aillon, K. L., Xie, Y., El-Gendy, N., Berkland, C. J. and Forrest, M. L. (2009). Effects of nanomaterial physicochemical properties on *in vivo* toxicity. *Advanced Drug Delivery Reviews, 61*(6), 457-466.

Alkilany, A. M. and Murphy, C. J. (2010). Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? *Journal of Nanoparticle Research, 12*(7), 2313-2333.

Ammendolia, M. G., Iosi, F., Maranghi, F., Tassinari, R., Cubadda, F., Aureli, F., Raggi, A., Superti, F., Mantovani, A. and De Berardis, B. (2017). Short-term oral exposure to low doses of nano-sized TiO_2 and potential modulatory effects on intestinal cells. *Food and Chemical Toxicology, 102*, 63-75.

Antony, J. J., Sivalingam, P. and Chen, B. (2015). Toxicological effects of silver nanoparticles. *Environmental Toxicology and Pharmacology, 40*(3), 729-732.

Aranda, A., Sequedo, L., Tolosa, L., Quintas, G., Burello, E., Castell, J. V. and Gombau, L. (2013). Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: A quantitative method for oxidative stress assessment of nanoparticle-treated cells. *Toxicology in Vitro, 27*(2), 954-963.

Arora, S., M., R. J. and Paknikar, K. M. (2012). Nanotoxicology and *in vitro* studies: The need of the hour. *Toxicology and Applied Pharmacology, 258*(2), 151-165.

AshaRani, P. V., Mun, G. L. K., Hande, M. P. and Valiyaveettil, S. (2009). Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells. *American Chemical Society Nano, 3*(2), 279-290.

Aueviriyavit, S., Phummiratch, D. and Maniratanachote, R. (2014). Mechanistic study on the biological effects of silver and gold nanoparticles in Caco-2 cells--induction of the Nrf2/HO-1 pathway by high concentrations of silver nanoparticles. *Toxicology Letters, 224*(1), 73-83.

Augustine, R., Hasan, A., Primavera, R., Wilson, R. J., Thakor, A. S. and Kevadiya, B. D. (2020). Cellular uptake and retention of nanoparticles: Insights on particle properties and interaction with cellular components. *Materials Today Communications, 25*, 101692.

Bailey, C. A., Bryla, P. and Malick, A. W. (1996). The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Advanced Drug Delivery Reviews, 22*(1), 85-103.

Bar-Ilan, O., Albrecht, R. M., Fako, V. E. and Furgeson, D. Y. (2009). Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. *Small, 5*(16), 1897-1910.

Barošová, H., Dvořáčková, J., Motyka, O., Kutláková, K. M., Peikertová, P., Rak, J., Bielniková, H. and Kukutschová, J. (2015). Metal-based particles in human amniotic fluids of fetuses with normal karyotype and congenital malformation-a pilot study. *Environmental Science and Pollution Research, 22*, 7582-7589.

Bayda, S., Adeel, M., Tuccinardi, T., Cordani, M. and Rizzolio, F. (2019). The History of Nanoscience and Nanotechnology: From Chemical-Physical Applications to Nanomedicine. *Molecules, 25*(1), 112.

Beddoes, C. M., Case, C. P. and Briscoe, W. H. (2015). Understanding nanoparticle cellular entry: A physicochemical perspective. *Advances in Colloid and Interface Science, 218*, 48-68.

Behra, R., Sigg, L., Clift, M. J., Herzog, F., Minghetti, M., Johnston, B., Petri-Fink, A. and Rothen-Rutishauser, B. (2013). Bioavailability of silver nanoparticles and ions: from a chemical and biochemical perspective. *Journal of the Royal Society Interface, 10*(87), 20130396.

Bergin, I. L. and Witzmann, F. A. (2013). Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps. *International journal of biomedical nanoscience and nanotechnology, 3*(1-2).

Bessa, M. J., Brandão, F., Viana, M., Gomes, J. F., Monfort, E., Cassee, F. R., Fraga, S. and Teixeira, J. P. (2020). Nanoparticle exposure and hazard in the ceramic

industry: an overview of potential sources, toxicity and health effects. *Environmental Research, 184*, 109297.

Bleeker, E. A. J., de Jong, W. H., Geertsma, R. E., Groenewold, M., Heugens, E. H. W., Koers-Jacquemijns, M., van de Meent, D., Popma, J. R., Rietveld, A. G., Wijnhoven, S. W. P., Cassee, F. R. and Oomen, A. G. (2013). Considerations on the EU definition of a nanomaterial: Science to support policy making. *Regulatory Toxicology and Pharmacology, 65*(1), 119-125.

Bongaerts, E., Nawrot, T. S., Van Pee, T., Ameloot, M. and Bové, H. (2020). Translocation of (ultra)fine particles and nanoparticles across the placenta; a systematic review on the evidence of *in vitro*, *ex vivo*, and *in vivo* studies. *Particle and fibre toxicology, 17*(1), 56.

Boverhof, D. R., Bramante, C. M., Butala, J. H., Clancy, S. F., Lafranconi, M., West, J. and Gordon, S. C. (2015). Comparative assessment of nanomaterial definitions and safety evaluation considerations. *Regulatory Toxicology and Pharmacology, 73*(1), 137-150.

Brar, S. K., Verma, M., Tyagi, R. D. and Surampalli, R. Y. (2010). Engineered nanoparticles in wastewater and wastewater sludge – Evidence and impacts. *Waste Management, 30*(3), 504-520.

Buchman, J. T., Hudson-Smith, N. V., Landy, K. M. and Haynes, C. L. (2019). Understanding Nanoparticle Toxicity Mechanisms To Inform Redesign Strategies To Reduce Environmental Impact. *Accounts of chemical research, 52*(6), 1632-1642.

Burdușel, A.-C., Gherasim, O., Grumezescu, A. M., Mogoantă, L., Ficai, A. and Andronescu, E. (2018). Biomedical Applications of Silver Nanoparticles: An Up-to-Date Overview. *Nanomaterials (Basel, Switzerland), 8*(9), 681.

Buzea, C., Pacheco, I. I. and Robbie, K. (2007). Nanomaterials and nanoparticles: Sources and toxicity. *Biointerphases*.

Calderón-Jiménez, B., Johnson, M. E., Bustos, A. R. M., Murphy, K. E., Winchester, M. R. and Baudrit, J. R. V. (2017). Silver Nanoparticles: Technological Advances, Societal Impacts, and Metrological Challenges. *Frontiers in Chemistry, 5*(6).

Carp, O., Huisman, C. L. and Reller, A. (2004). Photoinduced reactivity of titanium dioxide. *Progress in Solid State Chemistry, 32*(1), 33-177.

Carreira, S., Walker, L., Paul, K. and Saunders, M. (2015). The toxicity, transport and uptake of nanoparticles in the *in vitro* BeWo b30 placental cell barrier model used within NanoTEST. *Nanotoxicology, 9*, 66-78.

Cartwright, L., Poulsen, M. S., Nielsen, H. M., Pojana, G., Knudsen, L. E., Saunders, M. and Rytting, E. (2012). *In vitro* placental model optimization for nanoparticle transport studies. *International journal of nanomedicine, 7*, 497-510.

Cha, K., Hong, H. W., Choi, Y. G., Lee, M. J., Park, J. H., Chae, H. K., Ryu, G. and Myung, H. (2008). Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles. *Biotechnology Letters, 30*(11), 1893-1899.

Chen, N., Song, Z. M., Tang, H., Xi, W. S., Cao, A., Liu, Y. and Wang, H. (2016). Toxicological Effects of Caco-2 Cells Following Short-Term and Long-Term Exposure to Ag Nanoparticles. *International Journal of Molecular Sciences, 17*(6).

Choi, J.-Y., Ramachandran, G. and Kandlikar, M. (2009). The Impact of Toxicity Testing Costs on Nanomaterial Regulation. *Environmental Science & Technology, 43*(9), 3030-3034.

Commission, E. (2011). Definition of a nanomaterial. Retrieved from https://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm

De Matteis, V., Malvindi, M. A., Galeone, A., Brunetti, V., De Luca, E., Kote, S., Kshirsagar, P., Sabella, S., Bardi, G. and Pompa, P. P. (2015). Negligible particle-specific toxicity mechanism of silver nanoparticles: the role of Ag+ ion release in the cytosol. *Nanomedicine, 11*(3), 731-739.

Dimopoulou, M., Verhoef, A., Gomes, C. A., van Dongen, C. W., Rietjens, I. M. C. M., Piersma, A. H. and van Ravenzwaay, B. (2018). A comparison of the embryonic stem cell test and whole embryo culture assay combined with the BeWo placental passage model for predicting the embryotoxicity of azoles. *Toxicology Letters, 286*, 10-21.

Donaldson and Poland, C. A. (2013). Nanotoxicity: challenging the myth of nano-specific toxicity. *Current Opinion in Biotechnology, 24*(4), 724-734.

Dorier, M., Tisseyre, C., Dussert, F., Béal, D., Arnal, M.-E., Douki, T., Valdiglesias, V., Laffon, B., Fraga, S., Brandão, F., Herlin-Boime, N., Barreau, F., Rabilloud, T. and Carriere, M. (2019). Toxicological impact of acute exposure to E171 food additive and TiO2 nanoparticles on a co-culture of Caco-2 and HT29-MTX intestinal cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 845*, 402980.

Drasler, B., Sayre, P., Steinhäuser, K. G., Petri-Fink, A. and Rothen-Rutishauser, B. (2017). *In vitro* approaches to assess the hazard of nanomaterials. *NanoImpact, 8*, 99-116.

Ema, M., Kobayashi, N., Naya, M., Hanai, S. and Nakanishi, J. (2010). Reproductive and developmental toxicity studies of manufactured nanomaterials. *Reproductive Toxicology, 30*(3), 343-352.

Fisichella, M., Berenguer, F., Steinmetz, G., Auffan, M., Rose, J. and Prat, O. (2012). Intestinal toxicity evaluation of TiO_2 degraded surface-treated nanoparticles: a combined physico-chemical and toxicogenomics approach in caco-2 cells. *Particle and fibre toxicology, 9*, 18.

Fröhlich, E. (2018). Comparison of conventional and advanced in vitro models in the toxicity testing of nanoparticles. *Artificial Cells, Nanomedicine, and Biotechnology, 46*, 1091-1107.

Fröhlich, E. and Roblegg, E. (2012). Models for oral uptake of nanoparticles in consumer products. *Toxicology, 291*(1), 10-17.

Fry, R. C., Bangma, J., Szilagyi, J. and Rager, J. E. (2019). Developing novel *in vitro* methods for the risk assessment of developmental and placental toxicants in the environment. *Toxicology and Applied Pharmacology, 378*, 114635.

Gatoo, M. A., Naseem, S., Arfat, M. Y., Dar, A. M., Qasim, K. and Zubair, S. (2014). Physicochemical Properties of Nanomaterials: Implication in Associated Toxic Manifestations. *BioMed Research International, 2014*.

Gaumet, M., Gurny, R. and Delie, F. (2009). Localization and quantification of biodegradable particles in an intestinal cell model: the influence of particle size. *European Journal of Pharmaceutical Sciences, 36*(4-5), 465-473.

Gerloff, K., Fenoglio, I., Carella, E., Kolling, J., Albrecht, C., Boots, A. W., Förster, I. and Schins, R. P. F. (2012). Distinctive Toxicity of TiO_2 Rutile/Anatase Mixed Phase Nanoparticles on Caco-2 Cells. *Chemical Research in Toxicology, 25*(3), 646-655.

Gillois, K., Stoffels, C., Leveque, M., Fourquaux, I., Blesson, J., Mils, V., Cambier, S., Vignard, J., Terrisse, H., Mirey, G., Audinot, J.-N., Theodorou, V., Ropers, M.-H., Robert, H. and Mercier-Bonin, M. (2021). Repeated exposure of Caco-2 versus Caco-2/HT29-MTX intestinal cell models to (nano)silver *in vitro*: Comparison of two commercially available colloidal silver products. *Science of The Total Environment, 754*.

Gioria, S., Urbán, P., Hajduch, M., Barboro, P., Cabaleiro, N., La Spina, R. and Chassaigne, H. (2018). Proteomics study of silver nanoparticles on Caco-2 cells. *Toxicology in Vitro, 50*, 347-372.

Grafmüller, S., Manser, P., Krug, H. F., Wick, P. and von Mandach, U. (2013). Determination of the Transport Rate of Xenobiotics and Nanomaterials Across the Placenta using the *ex vivo* Human Placental Perfusion Model. *Journal of Visualized Experiments,*(76), e50401.

Gupta, R. and Xie, H. (2018). Nanoparticles in Daily Life: Applications, Toxicity and Regulations. *Journal of Environmental Pathology, Toxicology and Oncology, 37*(3), 209-230.

Gurunathan, S., Park, J. H., Han, J. W. and Kim, J. H. (2015). Comparative assessment of the apoptotic potential of silver nanoparticles synthesized by *Bacillus tequilensis* and *Calocybe indica* in MDA-MB-231 human breast cancer cells: targeting p53 for anticancer therapy. *International journal of nanomedicine, 10*, 4203-4223.

Hagens, W. I., Oomen, A. G., de Jong, W. H., Cassee, F. R. and Sips, A. J. (2007). What do we (need to) know about the kinetic properties of nanoparticles in the body? *Regulatory Toxicology and Pharmacology, 49*(3), 217-229.

Heringa, M. B., Geraets, L., van Eijkeren, J. C., Vandebriel, R. J., de Jong, W. H. and Oomen, A. G. (2016). Risk assessment of titanium dioxide nanoparticles via oral exposure, including toxicokinetic considerations. *Nanotoxicology, 10*(10), 1515-1525.

Hsin, Y. H., Chen, C. F., Huang, S., Shih, T. S., Lai, P. S. and Chueh, P. J. (2008). The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. *Toxicology Letters, 179*(3), 130-139.

Jamkhande, P. G., Ghule, N. W., Bamer, A. H. and Kalaskar, M. G. (2019). Metal nanoparticles synthesis: An overview on methods of preparation, advantages and disadvantages, and applications. *Journal of Drug Delivery Science and Technology, 53*, 101174.

Johnston, H. J., Hutchison, G., Christensen, F. M., Peters, S., Hankin, S. and Stone, V. (2010). A review of the *in vivo* and *in vitro* toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity. *Critical Reviews in Toxicology, 40*(4), 328-346.

Johnston, H. J., Hutchison, G. R., Christensen, F. M., Peters, S., Hankin, S. and Stone, V. (2009). Identification of the mechanisms that drive the toxicity of TiO_2 particulates: the contribution of physicochemical characteristics. *Particle and fibre toxicology, 6*, 33.

Jung, I., Joo, E. J., Suh, B. S., Ham, C. B., Han, J. M., Kim, Y. G., Yeom, J. S., Choi, J. Y. and Park, J. H. (2017). A case of generalized argyria presenting with muscle weakness. *Annals of Occupational and Environmental Medicine, 29*, 45.

Kang, T., Guan, R., Chen, X., Song, Y., Jiang, H. and Zhao, J. (2013). *In vitro* toxicity of different-sized ZnO nanoparticles in Caco-2 cells. *Nanoscale Research Letters, 8*(1), 496.

Karmakar, A., Zhang, Q. and Zhang, Y. (2014). Neurotoxicity of nanoscale materials. *Journal of Food and Drug Analysis, 22*(1), 147-160.

Khlebtsov, N. and Dykman, L. (2011). Biodistribution and toxicity of engineered gold nanoparticles: a review of *in vitro* and *in vivo* studies. *40*, 1647-1671.

Klasen, H. J. (2000). Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns, 26*(2), 117-130.

Koeneman, B. A., Zhang, Y., Westerhoff, P., Chen, Y., Crittenden, J. C. and Capco, D. G. (2010). Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. *Cell Biology and Toxicology, 26*(3), 225-238.

Kong, J., Franklin, N. R., Zhou, C., Chapline, M. G., Peng, S., Cho, K. and Dai, H. (2000). Nanotube Molecular Wires as Chemical Sensors. *Science, 287*(5453), 622-625.

Kroll, A., Pillukat, M. H., Hahn, D. and Schnekenburger, J. (2012). Interference of engineered nanoparticles with in vitro toxicity assays. *Archives of Toxicology, 86*(7), 1123-1136.

Kuhlbusch, T. A. J., Wijnhoven, S. W. P. and Haase, A. (2018). Nanomaterial exposures for worker, consumer and the general public. *NanoImpact, 10*, 11-25.

Lai, D. Y. (2012). Toward toxicity testing of nanomaterials in the 21st century: a paradigm for moving forward. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 4*(1), 1-15.

Landsiedel, R., Fabian, E., Ma-Hock, L., Wohlleben, W., Wiench, K., Oesch, F. and van Ravenzwaay, B. (2012). Toxico-/biokinetics of nanomaterials. *Archives of Toxicology, 86*(7), 1021-1060.

Lee, J. H., Mun, J., Park, J. D. and Yu, I. J. (2012). A health surveillance case study on workers who manufacture silver nanomaterials. *Nanotoxicology, 6*(6), 667-669.

Lefebvre, D. E., Venema, K., Gombau, L., Valerio, L. G., Raju, J., Bondy, G. S., Bouwmeester, H., Singh, R. P., Clippinger, A. J., Collnot, E.-M., Mehta, R. and Stone, V. (2015). Utility of models of the gastrointestinal tract for assessment of the digestion and absorption of engineered nanomaterials released from food matrices. *Nanotoxicology, 9*(4), 523-542.

Li, X., Liu, W., Sun, L., Aifantis, K. E., Yu, B., Fan, Y., Feng, Q., Cui, F. and Watari, F. (2015). Effects of physicochemical properties of nanomaterials on their toxicity. *Journal of Biomedical Materials Research Part A, 103*(7), 2499-2507.

Lima, R., Seabra, A. B. and Duran, N. (2012). Silver nanoparticles: a brief review of cytotoxicity and genotoxicity of chemically and biogenically synthesized nanoparticles. *Journal of Applied Toxicology, 32*(11), 867-879.

Liu, F., Mahmood, M., Xu, Y., Watanabe, F., Biris, A. S., Hansen, D. K., Inselman, A., Casciano, D., Patterson, T. A., Paule, M. G., Slikker, W., Jr. and Wang, C. (2015).

Effects of silver nanoparticles on human and rat embryonic neural stem cells. *Frontiers in Neuroscience, 9*, 115.

Lopez-Chaves, C., Soto-Alvaredo, J., Montes-Bayon, M., Bettmer, J., Llopis, J. and Sanchez-Gonzalez, C. (2018). Gold nanoparticles: Distribution, bioaccumulation and toxicity. *In vitro* and *in vivo* studies. *Nanomedicine: Nanotechnology, Biology and Medicine, 14*(1), 1-12.

Malakar, A., Kanel, S. R., Ray, C., Snow, D. D. and Nadagouda, M. N. (2021). Nanomaterials in the environment, human exposure pathway, and health effects: A review. *Science of The Total Environment, 759*, 143470.

Manangama, G., Migault, L., Audignon-Durand, S., Gramond, C., Zaros, C., Bouvier, G., Brochard, P., Sentilhes, L., Lacourt, A. and Delva, F. (2019). Maternal occupational exposures to nanoscale particles and small for gestational age outcome in the French Longitudinal Study of Children. *Environment International, 122*, 322-329.

Martirosyan, A., Bazes, A. and Schneider, Y.-J. (2014). *In vitro* toxicity assessment of silver nanoparticles in the presence of phenolic compounds – preventive agents against the harmful effect? *Nanotoxicology, 8*(5), 573-582.

Mathiesen, L., Mørck, T. A., Zuri, G., Andersen, M. H., Pehrson, C., Frederiksen, M., Mose, T., Rytting, E., Poulsen, M. S., Nielsen, J. K. and Knudsen, L. E. (2014). Modelling of Human Transplacental Transport as Performed in Copenhagen, Denmark. *Basic & Clinical Pharmacology & Toxicology, 115*(1), 93-100.

Maynard, A. D. and Aitken, R. J. (2007). Assessing exposure to airborne nanomaterials: Current abilities and future requirements. *Nanotoxicology, 1*(1), 26-41.

Maynard, A. D., Aitken, R. J., Butz, T., Colvin, V., Donaldson, K., Oberdörster, G., Philbert, M. A., Ryan, J., Seaton, A., Stone, V., Tinkle, S. S., Tran, L., Walker, N. J. and Warheit, D. B. (2006). Safe handling of nanotechnology. *Nature, 444*(7117), 267-269.

McShan, D., Ray, P. C. and Yu, H. (2014). Molecular Toxicity Mechanism of Nanosilver. *Journal of Food and Drug Analysis, 22*(1), 116-127.

Medici, S., Peana, M., Pelucelli, A. and Zoroddu, M. A. (2021). An updated overview on metal nanoparticles toxicity. *Semin Cancer Biol*.

Mei, N., Zhang, Y., Chen, Y., Guo, X., Ding, W., Ali, S. F., Biris, A. S., Rice, P., Moore, M. M. and Chen, T. (2012). Silver nanoparticle-induced mutations and oxidative stress in mouse lymphoma cells. *Environmental and Molecular Mutagenesis, 53*(6), 409-419.

Meng, H., Leong, W., Leong, K. W., Chen, C. and Zhao, Y. (2018). Walking the line: The fate of nanomaterials at biological barriers. *Biomaterials, 174*, 41-53.

Mesquita, B., Lopes, I., Silva, S., Bessa, M. J., Starykevich, M., Carneiro, J., Galvão, T. L. P., Ferreira, M. G. S., Tedim, J., Teixeira, J. P. and Fraga, S. (2017). Gold nanorods induce early embryonic developmental delay and lethality in zebrafish (*Danio rerio*). *Journal of Toxicology and Environmental Health, Part A*.

Moe, A. J. (1995). Placental amino acid transport. *American Journal of Physiology-Cell Physiology, 268*(6), 1321-1331.

Moreira, L., Costa, C., Pires, J., Teixeira, J. P. and Fraga, S. (2021). How can exposure to engineered nanomaterials influence our epigenetic code? A review of the mechanisms and molecular targets. *Mutation Research-Reviews in Mutation Research, 788*, 108385.

Mortensen, N. P., Caffaro, M. M., Patel, P. R., Uddin, M. J., Aravamudhan, S., Sumner, S. J. and Fennell, T. R. (2020). Investigation of twenty metal, metal oxide, and metal sulfide nanoparticles' impact on differentiated Caco-2 monolayer integrity. *NanoImpact*, 100212.

Myllynen, Loughran, M. J., Howard, C. V., Sormunen, R., Walsh, A. A. and Vähäkangas, K. H. (2008). Kinetics of gold nanoparticles in the human placenta. *Reproductive Toxicology, 26*(2), 130-137.

Myllynen, P. and Vähäkangas, K. (2013). Placental transfer and metabolism: an overview of the experimental models utilizing human placental tissue. *Toxicology in vitro : an international journal published in association with BIBRA, 27*(1), 507-512.

NanoToxClass. (2017). Standard Operation Procedure - Preparation of nanoparticle suspensions by cup horn sonication. *ERANET-SIINN Project NanoToxClass*.

Nel, A., Xia, T., Mädler, L. and Li, N. (2006). Toxic potential of materials at the nanolevel. *Science, 311*(5761), 622-627.

Oberdörster, G., Stone, V. and Donaldson, K. (2007). Toxicology of nanoparticles: A historical perspective. *Nanotoxicology, 1*(1), 2-25.

Pan, Y., Neuss, S., Leifert, A., Fischler, M., Wen, F., Simon, U., Schmid, G., Brandau, W. and Jahnen-Dechent, W. (2007). Size-dependent cytotoxicity of gold nanoparticles. *Small, 3*(11), 1941-1949.

Park, M. V., Neigh, A. M., Vermeulen, J. P., de la Fonteyne, L. J., Verharen, H. W., Briedé, J. J., van Loveren, H. and de Jong, W. H. (2011). The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles. *Biomaterials, 32*(36), 9810-9817.

Pattan, G. and Kaul, G. (2014). Health hazards associated with nanomaterials. *Toxicology and Industrial Health, 30*(6), 499-519.

Pedata, P., Ricci, G., Malorni, L., Venezia, A., Cammarota, M., Volpe, M. G., Iannaccone, N., Guida, V., Schiraldi, C., Romano, M. and Iacomino, G. (2019). *In vitro* intestinal

epithelium responses to titanium dioxide nanoparticles. *Food Research International, 119*, 634-642.

Peters, R. J. B., van Bemmel, G., Herrera-Rivera, Z., Helsper, H. P. F. G., Marvin, H. J. P., Weigel, S., Tromp, P. C., Oomen, A. G., Rietveld, A. G. and Bouwmeester, H. (2014). Characterization of Titanium Dioxide Nanoparticles in Food Products: Analytical Methods To Define Nanoparticles. *Journal of Agricultural and Food Chemistry, 62*(27), 6285-6293.

Pietroiusti, A., Magrini, A. and Campagnolo, L. (2014). 2 - Mechanisms of nanomaterial toxicity. In Njuguna, J., Pielichowski, K. and Zhu, H. *Health and Environmental Safety of Nanomaterials* (28-43): Woodhead Publishing.

Pietroiusti, A., Stockmann-Juvala, H., Lucaroni, F. and Savolainen, K. (2018). Nanomaterial exposure, toxicity, and impact on human health. *Wiley Interdisciplinary Reviews - Nanomedicine and Nanobiotechnology, 10*, e1513.

Pietroiusti, Antonio, Campagnolo, L. and Fadeel, B. (2013). Interactions of Engineered Nanoparticles with Organs Protected by Internal Biological Barriers. *Small, 9*(9-10), 1557-1572.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D. and Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity, 2017*, 8416763.

Ponti, J., Colognato, R., Franchini, F., Gioria, S., Simonelli, F., Abbas, K., Uboldi, C., Kirkpatrick, C. J., Holzwarth, U. and Rossi, F. (2009). A quantitative *in vitro* approach to study the intracellular fate of gold nanoparticles: from synthesis to cytotoxicity. *Nanotoxicology, 3*(4), 296-306.

Poulsen, M. S., Mose, T., Maroun, L. L., Mathiesen, L., Knudsen, L. E. and Rytting, E. (2015). Kinetics of silica nanoparticles in the human placenta. *Nanotoxicology, 9 (0 1)*, 79-86.

Prouillac, C. and Lecoeur, S. (2010). The Role of the Placenta in Fetal Exposure to Xenobiotics: Importance of Membrane Transporters and Human Models for Transfer Studies. *Drug Metabolism and Disposition, 38*(10), 1623-1635.

Pulskamp, K., Diabaté, S. and Krug, H. F. (2007). Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicol Lett, 168*(1), 58-74.

Rahman, M. F., Wang, J., Patterson, T. A., Saini, U. T., Robinson, B. L., Newport, G. D., Murdock, R. C., Schlager, J. J., Hussain, S. M. and Ali, S. F. (2009). Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles. *Toxicology Letters, 187*(1), 15-21.

Ramos, A. P., Cruz, M. A. E., Tovani, C. B. and Ciancaglini, P. (2017). Biomedical applications of nanotechnology. *Biophysical reviews, 9*(2), 79-89.

Rattanapinyopituk, K., Shimada, A., Morita, T., Sakurai, M., Asano, A., Hasegawa, T., Inoue, K. and Takano, H. (2014). Demonstration of the Clathrin- and Caveolin-Mediated Endocytosis at the Maternal–Fetal Barrier in Mouse Placenta after Intravenous Administration of Gold Nanoparticles. *Journal of Veterinary Medical Science, 76*(3), 377-387.

Regulation, C. (2019). Annex VI to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. *Official Journal of the European Union*.

Reidy, B., Haase, A., Luch, A., Dawson, K. A. and Lynch, I. (2013). Mechanisms of Silver Nanoparticle Release, Transformation and Toxicity: A Critical Review of Current Knowledge and Recommendations for Future Studies and Applications. *Materials, 6*(6), 2295-2350.

Rothbauer, M., Patel, N., Gondola, H., Siwetz, M., Huppertz, B. and Ertl, P. (2017). A comparative study of five physiological key parameters between four different human trophoblast-derived cell lines. *Scientific Reports, 7*(1), 5892.

Sanchez, V. C., Jachak, A., Hurt, R. H. and Kane, A. B. (2012). Biological interactions of graphene-family nanomaterials: an interdisciplinary review. *Chemical Research in Toxicology, 25*(1), 15-34.

Sani, A., Cao, C. and Cui, D. (2021). Toxicity of gold nanoparticles (AuNPs): A review. *Biochemistry and Biophysics Reports, 26*, 100991.

Saratale, R. G., Karuppusamy, I., Saratale, G. D., Pugazhendhi, A., Kumar, G., Park, Y., Ghodake, G. S., Bharagava, R. N., Banu, J. R. and Shin, H. S. (2018). A comprehensive review on green nanomaterials using biological systems: Recent perception and their future applications. *Colloids and Surfaces B: Biointerfaces, 170*, 20-35.

Schlinkert, P., Casals, E., Boyles, M., Tischler, U., Hornig, E., Tran, N., Zhao, J., Himly, M., Riediker, M., Oostingh, G. J., Puntes, V. and Duschl, A. (2015). The oxidative potential of differently charged silver and gold nanoparticles on three human lung epithelial cell types. *Journal of Nanobiotechnology, 13*, 1.

Schrand, A. M., Rahman, M. F., Hussain, S. M., Schlager, J. J., Smith, D. A. and Syed, A. F. (2010). Metal-based nanoparticles and their toxicity assessment. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2*(5), 544-568.

Shi, H., Magaye, R., Castranova, V. and Zhao, J. (2013). Titanium dioxide nanoparticles: a review of current toxicological data. *Particle and fibre toxicology, 10*, 15-15.

Song, Y., Guan, R., Lyu, F., Kang, T., Wu, Y. and Chen, X. (2014). *In vitro* cytotoxicity of silver nanoparticles and zinc oxide nanoparticles to human epithelial colorectal adenocarcinoma (Caco-2) cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 769*, 113-118.

Stone, V., Miller, M. R., Clift, M. J. D., Elder, A., Mills, N. L., Møller, P., Schins, R. P. F., Vogel, U., Kreyling, W. G., Alstrup Jensen, K., Kuhlbusch, T. A. J., Schwarze, P. E., Hoet, P., Pietroiusti, A., De Vizcaya-Ruiz, A., Baeza-Squiban, A., Teixeira, J. P., Tran, C. L. and Cassee, F. R. (2017). Nanomaterials Versus Ambient Ultrafine Particles: An Opportunity to Exchange Toxicology Knowledge. *Environmental Health Perspectives, 125*(10), 106002.

Tassinari, R., Cubadda, F., Moracci, G., Aureli, F., D'Amato, M., Valeri, M., De Berardis, B., Raggi, A., Mantovani, A., Passeri, D., Rossi, M. and 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*(6), 654-662.

Tucci, P., Porta, G., Agostini, M., Dinsdale, D., Iavicoli, I., Cain, K., Finazzi-Agró, A., Melino, G. and Willis, A. (2013). Metabolic effects of TiO_2 nanoparticles, a common component of sunscreens and cosmetics, on human keratinocytes. *Cell Death & Disease, 4*(3), e549.

Uz, M., Bulmus, V. and Alsoy Altinkaya, S. (2016). Effect of PEG Grafting Density and Hydrodynamic Volume on Gold Nanoparticle-Cell Interactions: An Investigation on Cell Cycle, Apoptosis, and DNA Damage. *Langmuir, 32*(23), 5997-6009.

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, 38*(9), 1195-1205.

Vinith, M., Antoine, M. and Jeffrey, A. K. (2011). Nanoparticulate Drug Delivery in Pregnancy: Placental Passage and Fetal Exposure. *Current Pharmaceutical Biotechnology, 12*(5), 731-742.

Wright, S. L. and Kelly, F. J. (2017). Plastic and Human Health: A Micro Issue? *Environmental Science & Technology, 51*(12), 6634-6647.

Xu, Y., Shrestha, N., Préat, V. and Beloqui, A. (2021). An overview of in vitro, ex vivo and in vivo models for studying the transport of drugs across intestinal barriers. *Advanced Drug Delivery Reviews, 175*, 113795.

Yamashita, K., Yoshioka, Y., Higashisaka, K., Mimura, K., Morishita, Y., Nozaki, M., Yoshida, T., Ogura, T., Nabeshi, H., Nagano, K., Abe, Y., Kamada, H., Monobe, Y., Imazawa, T., Aoshima, H., Shishido, K., Kawai, Y., Mayumi, T., Tsunoda, S.-I.

and Tsutsumi, Y. (2011). Silica and Titanium Dioxide Nanoparticles Cause Pregnancy Complications in Mice. *Nature Nanotechnology, 6*, 321-328.

Young-Man, C., Yasuko, M., Jun-ichi, A., Takeshi, T., Mizuki, S. and Kumiko, O. (2018). Size-dependent acute toxicity of silver nanoparticles in mice. *Journal of Toxicologic Pathology, 31*(1), 73-80.

Zamaraeva, M. V., Sabirov, R. Z., Maeno, E., Ando-Akatsuka, Y., Bessonova, S. V. and Okada, Y. (2005). Cells die with increased cytosolic ATP during apoptosis: a bioluminescence study with intracellular luciferase. *Cell Death and Differentiation, 12*(11), 1390-1397.

Zhang, J., Song, W., Guo, J., Zhang, J., Sun, Z., Li, L., Ding, F. and Gao, M. (2013). Cytotoxicity of different sized TiO_2 nanoparticles in mouse macrophages. *Toxicology and Industrial Health, 29*(6), 523-533.