



Nanomedicine and epigenome. Possible health risks



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ABSTRACT

Nanomedicine is an emerging field that combines knowledge of nanotechnology and material science with pharmaceutical and biomedical sciences, aiming to develop nanodrugs with increased efficacy and safety. Compared to conventional therapeutics, nanodrugs manifest higher stability and circulation time, reduced toxicity and improved targeted delivery. Despite the obvious benefit, the accumulation of imaging agents and nanocarriers in the body following their therapeutic or diagnostic application generates concerns about their safety for human health. Numerous toxicology studies have demonstrated that exposure to nanomaterials (NMs) might pose serious risks to humans. Epigenetic modifications, representing a non-genotoxic mechanism of toxicant-induced health effects, are becoming recognized as playing a potential causative role in the aetiology of many diseases including cancer. This review i) provides an overview of recent advances in medical applications of NMs and ii) summarizes current evidence on their possible epigenetic toxicity. To discern potential health risks of NMs, since current data are mostly based upon *in vitro* and animal models, a better understanding of functional relationships between NM exposure, epigenetic deregulation and phenotype is required.

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1. Introduction

Nanomedicine, as an interdisciplinary science, combines the knowledge of molecular biology, pharmaceuticals, medicine, material science, engineering, and information technology. The application of nanotechnology to medicine provides an opportunity to study the biological systems at a more subtle level, giving rise to better understanding of disease mechanisms. Moreover, it enables more accurate and rapid diagnosis, targeted and effective drug delivery, novel ways of organ and tissue regeneration, and follow-up of diseases (Pablico-Lansigan et al., 2013; Schütz et al., 2013a,b; Chaudhury et al., 2014; Juillerat-Jeanneret et al., 2015).

In 2011 the European Commission published a recommendation on the definition of a nanomaterial (NM) as a material, sized in at least one dimension between 1 and 100 nm (http://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm). Nanoparticles (NPs) are NMs that have all three dimensions between 1 and 100 nm. A material can also be considered as a NM if its volume-specific surface area is larger than 60 m²cm⁻³ (Rauscher et al., 2017). However, the term NM in nanomedicine extends this

commonly accepted definition to particles with dimensions up to 1000 nm (Schütz et al., 2013a).

The biocompatibility and stability of NMs in physiological solutions is imperative for their development for clinical use (Mu et al., 2014). Entering the body, the surface of NMs is rapidly covered by proteins, resulting in formation of a 'corona' (Monopoli et al., 2012) which affects the distribution, pharmacokinetics, and circulation time of NMs in the body. Although it is impossible to completely avoid the formation of this protein layer (Tenzer et al., 2011) its composition can be altered through surface modification of NMs. Targeting of current nanodrugs has relied on the enhanced permeability and retention effect (Maeda, 2015). Functionalization of NMs (binding of specific ligands, antigen, aptamer, protein etc. on the surface of NMs) increases their accumulation in, e.g. a tumor region, an ischemic tissue, or organ inflamed area (Albanese et al., 2012). A controlled release of drugs can be triggered by pH (Sato et al., 2011), redox potential (Luo et al., 2011), presence of certain enzymes (De La Rica et al., 2012), and temperature (Kim and Lee, 2004). NMs are able to bypass biological barriers, such as cell membranes and the blood–brain barrier, allowing delivery of high drug concentrations in the target tissue. Particle size, shape, and surface chemistry are key factors that determine cellular uptake, biodistribution patterns, and clearance mechanisms (Nel et al., 2009; Treuel and Nienhaus, 2012). Modification of some of these

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features can induce new functions, and potential toxicity (Rahman et al., 2013).

Oxidative stress, one of the main mechanisms underlying NM-induced toxicity, is closely associated with inflammatory cell responses, immunotoxicity and also genotoxicity (Dusinska et al., 2015). For the safety assessment of NMs, genotoxicity testing is essential, as it addresses both potential mutagenicity and carcinogenicity. Apart from geno- and immunotoxicity, NMs may cause changes in epigenetic regulatory mechanisms that have been involved in the pathogenesis of several complex diseases including cancer (Stoccoro et al., 2013). Although research on NM-induced epigenetic alterations has increased significantly in recent years, the evidence on epigenetic toxicity of NMs is still mostly based on *in vitro* and animal models. To evaluate the impact of NMs on epigenetic deregulation is not a simple task due to multiple layers of epigenetic control mechanisms and large differences in individual susceptibility. All these and other limitations currently hamper incorporation of epigenetic toxicity endpoints in the standard battery of NM safety assessment.

This article provides a comprehensive review of current knowledge on epigenetic toxicity of NMs, with the focus on their nanomedical applications. Our aim is to summarize available data on epigenetic changes induced by NMs. To discern potential health risks, a knowledge of functional relationships between individual NM-induced epigenetic deregulation and phenotypic response is required.

2. Uses of NMs in nanomedicine

Application of the knowledge and tools of nanotechnology in medicine offers new possibilities in medical imaging and diagnosis, development of more powerful nanodrugs (both therapeutic and imaging agents), implantable materials, cancer treatment and tissue regeneration. More than 50 FDA-approved nanodrugs are currently in clinical use (Table 1) and more than 77 nano-based products are being evaluated in Phase I – III clinical trials (Bobo et al., 2016).

2.1. Diagnostics

Early and highly accurate disease diagnosis is a prerequisite for effective treatment and is an integral part of clinical medicine. Nanotechnology has introduced a number of NMs that have expanded the potential of targeted diagnostic imaging.

2.1.1. Imaging

NMs as a new and exciting class of imaging agents can be used for both anatomic and molecular imaging. Their small size and unique physicochemical properties offer intense, non-invasive and longitudinally stable imaging signals, high avidity (a large association constant brought about by the presence of multiple ligands per particle), multimodal signal capabilities (detection of one NP by more than one imaging modality, allowing deep tissue screening), multiplexing (detection of various molecular targets simultaneously) and moreover, theranostic capabilities (use for both diagnostic and therapeutic purposes) (Thakor et al., 2016).

Several commercially available dextran-coated superparamagnetic iron oxide NPs (SPIONs) have been approved as intravenous contrast agents for *in vivo* magnetic resonance imaging (MRI) (e.g. Endorem, Sinerem, Resovist) and several others are under clinical investigation (Etheridge et al., 2013; Thakor et al., 2016). Although these novel contrast agents present the advantages of suitable magnetic saturation, superparamagnetic properties, and colloidal stability, they have been taken off the market and are no longer manufactured due to concerns about their toxicity

and fatal anaphylactic reactions (Mahmoudi et al., 2008, 2009).

Several other NMs have been designed and applied as contrast enhancers in various optical imaging techniques. Quantum dots (QDs), carbon dots and carbonaceous NMs, noble metal NMs (mainly gold (Au) and silver (Ag)), organically modified fluorescently-doped silica, and various other NMs are promising optical (fluorescent) tools for cell tracking and visualization (Wolfbeis, 2015). The *in vivo* monitoring of NM-labelled therapeutic cells (mainly stem cells, SCs) by non-invasive imaging is crucial in order to assess their safety, efficacy and mechanisms of action. Several clinical trials have already been undertaken to assess the potential of mesenchymal stem/stromal cells (MSCs) to ameliorate kidney disease in cardiac surgery patients (NCT00733876; NCT01602328), cancer patients receiving cisplatin (NCT01275612), and to evaluate the safety and efficacy of bone marrow-derived mononuclear cells in patients with focal segmental glomerulosclerosis (NCT02693366) (Sharkey et al., 2016).

2.1.2. Medical biosensors

Nanobiosensors for molecular diagnostics represent an interesting application of the nanotechnology in medicine. The progress in molecular biology and 'omics' technologies allowed identification of key molecules having a causal role in pathogenesis of various diseases. Several NM-based biosensors have been developed for early detection of neurodegenerative diseases such as Alzheimer's disease (AD) (Perry et al., 2009), Creutzfeldt-Jacob disease (Kouassi et al., 2007) or Parkinson's disease (PD) (Alarcón-Angeles et al., 2008). There are currently several nanobiosensors for cardiovascular diseases using gold NPs (AuNPs) or silicon nanowires (Pavlov et al., 2004; Chua et al., 2009) and many others, for example, for cancers (HER2, EGFR, CA15-3, PSA, CEA etc.) are currently under development (Ge et al., 2014).

2.2. Regenerative medicine

Regenerative medicine is a broad interdisciplinary science that attempts to restore lost, damaged, or aging cells and tissues to a state as close as possible to their native architecture and function. Regenerative strategies include stem cell-based therapies and tissue engineering applications. NMs can play an important role in nanopatterning of implant surfaces and as 3D scaffolds mimicking the natural environment of cells, facilitating their mobility, adhesion, and differentiation (Engel et al., 2008; Zhang and Webster, 2009). Nano-structured poly(lactic-co-glycolic acid) (PLGA) surfaces have been shown to accelerate chondrocyte adhesion and proliferation (Savaiano and Webster, 2004; Park et al., 2005) and titanium surface improve endothelial cell functions (Lu et al., 2008). As the regenerative capability of the central nervous system (CNS) is limited, cellular-based therapies have emerged as a promising route of therapy for CNS-related diseases and injuries (Fischbach et al., 2013).

2.2.1. Bone regeneration

A wide range of nano- and micro-scale materials have been utilized in bone tissue engineering, including natural polymers such as collagen, gelatin, chitosan, alginates and silk and synthetic polymers such as hydroxyapatite (HA), poly(lactic acid) (PLA), PLGA, poly-epsilon-caprolactone, or polyvinyl alcohol (Liu and Ma, 2004; Perán et al., 2012), ceramics (Seitz et al., 2005; Gerhardt and Boccaccini, 2010) composites (Rezwan et al., 2006; Mittal et al., 2015), metals (Wen et al., 2002), and carbon nanostructure-reinforced composites (Perkins and Naderi, 2016). Nanofibrous matrices have also been shown to enhance the expression of osteogenic genes and proteins (Smith et al., 2009).

Table 1
Examples of approved and currently used products in nanomedicine.

Name	Description	Indication	Advantage	Application route	FDA approval year
Polymer-based NMs					
Adagen [®]	PEGylated adenosine deaminase enzyme	Severe combined immunodeficiency disease (SCID)	Improved circulation time and reduced immunogenicity	Intravenous	1990
Copaxone [®]	Random co-polymer of L-glutamate, L-alanine, L-lysine and L-tyrosine	Multiple Sclerosis (MS)	Based on its resemblance to myelin basic protein, it thought to divert as a “decoy” an autoimmune response against myelin	subcutaneous	1996
Macugen [®]	PEGylated anti-VEGF aptamer	Neovascular age-related macular degeneration	Improved stability of aptamer	intravitreal	2004
Mircera [®]	PEGylated epoetin β (erythropoietin receptor activator)	Anemia associated with chronic renal failure	Improved stability of protein	intravenous	2007
Neulasta [®]	PEGylated filgrastim (granulocytes colony stimulating factor)	Fibrile neutropenia, chemotherapy induced	Improved stability of protein	subcutaneous	2002
Oncospar [®]	PEGylated L-asparaginase	Acute lymphoblastic leukemia	Improved stability of protein	subcutaneous	1994
PegIntron [®]	PEGylated IFN α -2b protein	Hepatitis C	Improved stability of protein	subcutaneous	2001
Somavert [®]	PEGylated human growth hormone receptor antagonist	Acromegaly	Improved stability of protein	subcutaneous	2003
Lipid-based NMs					
Abelcet [®] / Amphocil	Liposomal amphotericin B lipid complex	Fungal infections	Reduced toxicity	intravenous	1995
AmBisome [®]	Liposomal amphotericin B	Fungal infections	Reduced nephrotoxicity	intravenous	1997
Doxil [®] /Caelyx [™]	Liposomal doxorubicin	Kaposi's sarcoma; ovarian cancer; multiple myeloma	Improved delivery to site of disease; decreased systemic toxicity	intravenous	1995 2005 2008
DepoCyt	Liposomal Cytarabine	Lymphomatous meningitis	Increased delivery to tumor site; lower systemic toxicity	intravenous	1999
Marqibo [®]	Liposomal vincristine	Acute lymphoblastic leukemia	Increased delivery to tumor site; lower systemic toxicity	intravenous	2012
Protein-drug conjugates					
Abraxane [®]	Albumine-bound paclitaxel NPs	Breast cancer; NSCLC (non-small-cell lung cancer) Pancreatic cancer	Improved solubility; improved delivery to tumor	intravenous	2005 2012 2013
Ontak [®]	Recombinant fusion protein of fragment A of diphtheria toxin and subunit binding to interleukin-2 receptor	Primary cutaneous T-cell lymphoma;	Targeted T-cell specificity; lysosomal escape	intravenous	1999
Nanocrystals					
Emend [®]	aprepitant	Antiemetic	Increased bioavailability due to increased dissolution rate	Oral capsule	2003
Rapamune [®]	sirolimus	Immunosuppressant for kidney transplants	Increased bioavailability	Oral tablet/ solution	1999
Micellar NMs combined with drug					
Estrasorb [®]	Emulsion of estradiol in soybean oil, polysorbate 80, ethanol and water	Hormone replacement therapy during menopause	Controlled delivery of therapeutic	transdermal	2003
Metal-based NMs					
Feraheme [®]	SPIONs coated with dextran	Iron deficiency anemia in adults with chronic kidney disease (CDK)	Magnetite suspension allows for prolonged steady release, decreasing number of doses	intravenous	2009
Venofer	Iron sucrose	Iron deficiency in chronic kidney disease (CDK)	Allow increased dose	intravenous	2000
Other nano-based products					
Vitoss [®]	Calcium phosphate	Bone substitute	Mimics bone structure allowing cell adhesion and growth	–	2003 ^a
Ostim [®]	hydroxyapatite	Bone substitute	Mimics bone structure allowing cell adhesion and growth	–	2004 ^a
Filtek [™]	Silica and zirconium NMs	Dental composite	Dental restoration/filling material	–	2008 ^a
Ceram [™] X duo	Ceramic NPs	Dental composite	Dental restoration/filling material	–	2005 ^a
Acticoat [®]	Antimicrobial nanosilver	Medical dressing	Antibacterial barrier	–	2005 ^a
Helixone [®]	Nanoporous membrane	Dialysis filter	Minimize diffusion resistance	–	1998 ^a
TiMESH	polypropylene with covalently bonded titanized surface (30-nm TiO ₂)	Tissue scaffold	Outstanding biocompatibility	–	2004 ^a

^a FDA approved through the 510(k) process (Zuckerman et al., 2011).

2.2.2. Cartilage regeneration

Cartilage is an avascular tissue composed of chondrocytes entrapped in an extracellular matrix (ECM) rich in proteoglycans and collagens; therefore, current reconstructive options for

cartilage repair are limited (Swieszkowski et al., 2007; Kumar et al., 2016). Tissue engineering strategies have been based mostly on matrix seeded with either chondrocytes or MSCs (Li et al., 2005). Biomaterials, such as collagen, fibrin, alginate, chitosan, hyaluronic

acid and polyesters have been incorporated into 3D exogenous ECMs for guiding cartilage regeneration (Vinatier et al., 2009). To promote cell attachment to scaffold matrices of implants, surface topography could be modified by nanolithography (Fiedler et al., 2013). A common problem with prosthesis is bacterial adhesion to implants (Costerton et al., 2005). To prevent pathogen adhesion, nanostructured titanium surfaces could be utilized (Singh et al., 2011).

2.2.3. Dentistry

Nanodentistry is an emerging field with significant potential to yield new generation of technologically advanced clinical tools and devices for oral healthcare (Besinis et al., 2015; Neel et al., 2015).

2.2.3.1. Dental caries management. Multiple innovative applications of nanotechnology have been postulated with the aim of attaining net remineralization of enamel as a non-invasive approach for dental caries management (ten Cate, 2012). NMs used in the field of remineralization comprised nanosilver fluoride, nanosized calcium fluoride, carbonate-HA nanocrystals, nanoparticulate HA, nanosized amorphous calcium phosphate particles, casein phosphopeptide-amorphous calcium phosphate nanocomplexes, nanoparticulate tricalcium phosphate, and bioactive glass NMs (Elkassas and Arafa, 2017).

2.2.3.2. Dental implants. Nanoscale modification of dental implant surfaces to improve recruitment and migration of osteoblasts includes both surface topography as well as chemistry (Ogawa et al., 2008; Brammer et al., 2009). Nanoscale deposits of HA and calcium phosphate create a more complex implant surface for osteogenesis (Goené et al., 2007). Commercial nanoproducts used to treat bone defects are Ostim[®] (Heraeus Kulzer, Hanau, Germany), VITOSSO (Orthovita, Inc, USA and NanOSS[™] (Angstrom Medica, USA) (Kumar and Vijayalakshmi, 2006; Machot et al., 2014).

2.2.4. Cell encapsulation

Cell encapsulation, i.e. the immobilization of cells within polymeric microspheres or microcapsules, has permitted the transplantation of cells into human and animal subjects without the need for immunosuppressants, while allowing the bidirectional diffusion of nutrients, oxygen and waste (Murua et al., 2008). Microencapsulation materials have comprised natural or synthetic polymers or blends, including alginate, collagen, gelatin, fibrin, polyphosphazenes, poly(acrylic acids), poly(methacrylic acids), copolymers of acrylic acid and methacrylic acid, poly(alkylene oxides), poly(vinyl acetate), polyvinylpyrrolidone, PEG, polyethersulfone, polysaccharides such as agarose, cellulose sulfate, chondroitin sulfate, chitosan, hyaluronan, and copolymers, and blends of each (Rabanel et al., 2009). The first implantable alginate-poly(L-lysine) microcapsules for the treatment of diabetes were presented by Lim and Sun, (1980). The encapsulation systems under clinical testing (Phase I/II clinical trials) to treat diabetes have demonstrated good safety profiles (Desai and Shea, 2016). In addition, many other microencapsulated cell systems are now being investigated clinically to treat hypothyroidism; atrophic macular degeneration or retinitis pigmentosa; Huntington's disease; AD; pancreatic cancer; and stroke (Olabisi, 2015; Iacovacci et al., 2016). The encapsulation of local anesthetics into nanoliposomes is used to produce a system for prolonged drug release (Vahabi and Eatemadi, 2017).

2.3. Therapy

In the past decades, a variety of nanoscale therapeutic systems have been developed and some of them have been employed in

clinical diagnosis and therapy. They have gained a growing interest because of improved half-life in blood circulation, enhanced drug bioavailability, fewer side effects and better synergistic outcomes compared to conventional chemotherapeutic agents (Peng et al., 2015; Zang et al., 2017).

2.3.1. Drug delivery

Nanocarriers are broadly classified into three major categories: i) polymer-based nanocarriers, ii) lipid-based nanocarriers and iii) inorganic platforms (Peng et al., 2015).

Polymer-based nanocarriers is a collective term given to any type of polymer NM, but specifically nanospheres and nanocapsules. Synthetic biodegradable polymeric NMs include poly(ϵ -caprolactone), PLA, PLGA, polyglycolic acid, and poly(alkylcyanoacrylate). Natural polymers include gelatin, dextran ester, and chitosan; however, they do not have as high purity and reproducibility as the synthetic polymers. PLA and PLGA are FDA-approved biodegradable and biocompatible NMs especially for cancer-related human applications (Faraji and Wipf, 2009; Khan et al., 2016). Since approval of Abraxane (albumin based drug delivery vehicle containing paclitaxel) for the treatment of patients with metastatic breast cancer by the FDA, there is increased interest in the fabrication of similar protein-based nanocarriers in cancer treatment (Bobo et al., 2016). A polymeric NM formulation of docetaxel is currently under phase 1 clinical trials in patients with advanced solid malignancies (NCT00103791). Natural polysaccharides such as chitosan have been widely used in the fabrication of nanocarriers for delivery of various therapeutic agents including proteins, drugs, and genes (Swierczewska et al., 2016). Immense attention has been given to the self-assembly of amphiphilic block copolymers (comprising a hydrophilic head-group such as PEG, and a hydrophobic tail such as polybutylene), for loading hydrophobic drugs/gene/phototherapeutic agents (He et al., 2016).

Lipid-based nanocarriers, lipid NMs or liposomes are made from a self-assembling concentric lipid bilayer that is primarily composed of amphipathic phospholipids enclosing an interior aqueous space. Liposomes are highly biocompatible nanocarriers comprised of extremely cheap raw materials (soybean oil, lecithin etc.) that permit the use of the simplest fabrication methods (Texier et al., 2009). Doxil[®] (DOX-containing PEGylated liposome) was the first therapeutic liposomal delivery vehicle approved by the FDA in 1995 (Jain and Stylianopoulos, 2010). Since then, liposomal drug delivery systems are at the leading-edge of nanoscale drug delivery platform, because of their unique abilities to accommodate both therapeutic and diagnostic agents in a single closed lipid bilayer, made of either synthetic or natural phospholipids (Lajunen et al., 2016).

Inorganic nanocarriers such as metals, metal oxides, metal sulfides etc. have gained prominent attention only in the recent few years (Huang et al., 2011). In contrast to organic NMs, inorganic NMs possess optical and magnetic properties coupled with other unique physical characteristics such as inertness, stability, and ease of functionalization that makes them superior in cancer imaging and therapy. They are relatively stable over large ranges of pH and temperatures; however, their lack of biodegradation and slow dissolution rates raise concern and uncertainty regarding their degradation and elimination from the body. Noble metals such as Au and Ag are recognized for their unique surface plasmon resonance properties, that can dramatically intensify the emitted light (or metal enhanced fluorescence) (Kochuveedu and Kim, 2014). Mesoporous silica (NMs) have a promising advantage in cancer theranostics (Liu et al., 2016a). High surface area and pore volume are suitable for higher payloads, while surface enriched silanol groups allow conjugation of targeting ligands for targeted cancer therapy (Yang and Yu, 2016). Carbon nanotubes (CNTs) are being

investigated for their use in gene and drug delivery, since they can readily cross biological barriers (Thakor and Gambhir, 2013).

2.3.2. Phototherapy (photothermal and photodynamic therapy)

Phototherapy, an emerging non-invasive technique, has recently emerged as a viable therapeutic option in the treatment of cancer. The principle of this treatment modality is deployment of heat (photothermal therapy, PTT) or singlet oxygen (photodynamic therapy, PDT) generated from irradiated light to destroy cancer cells with high efficiency (Liu et al., 2016a). While cells exposed to local hyperthermia (above 42 °C) induced by light irradiation are killed, there is only a negligible damage to the surrounding normal tissue and cellular components (Tang and McGoron, 2009). Temperature above 45 °C can cause direct cell death (ie, thermoablation) (Hildebrandt et al., 2002; Alexis et al., 2010).

PTT agents need to have an enhanced light absorption and efficient light-to-heat conversions. Promising PTT agents are noble metal NMs (e.g. Au nanospheres, nanorods, nanoshells, and nanocages, as well as Ag and Pd), carbon-based NMs (e.g. carbon spheres, CNTs, graphene oxide), nanoscale metal chalcogenides (e.g. Cu_{2-x}E, E = S, Se, and Te), transition metal dichalcogenide nanostructures (e.g. WS₂, MoS₂, WSe₂), or metal oxide NPs (e.g. WO_{3-x}, MoO_{3-x}) (Jain et al., 2008; Cheng et al., 2014; Jaque et al., 2014). Early clinical trials are currently underway using the near-infrared (NIR) PTT for refractory head and neck cancers with AuroShell NMs (NCT00848042) (Thakor and Gambhir, 2013). SPIONs have also been shown to generate heat when injected directly into tumors in the presence of an external magnetic field (Johannsen et al., 2007). In animal models of prostate cancer (Johannsen et al., 2004), malignant glioma (Jordan et al., 2006), and breast cancer (Jordan et al., 1997) magnetic fluid hyperthermia has shown promising results. Phase 1 clinical trials for prostate cancer and phase 2 for brain cancer are currently underway (Alexis et al., 2010).

PDT uses a light-activatable chemical, known as a photosensitizer, which absorbs light energy and transfers it to oxygen molecules generating cytotoxic reactive oxygen species. The effectiveness of PDT depends largely on the efficiency of the photosensitizers and their selective delivery to the target tumor tissue (Chatterjee et al., 2008). PDT and PTT combined with imaging to treat tumors with greater specificity and sensitivity has received significant attention (Gong et al., 2014; Liu et al., 2016b; Ma et al., 2016b). CNTs have been studied for photoacoustic and optical imaging since they have a strong optical absorbance in the NIR region that makes them ideal for NIR photothermal ablation therapy.

2.3.3. Immunotherapy

The use of NMs to target the immune system is an intensely active area of research and development. NM-based immunotherapy represents a novel approach for cancer treatment, autoimmune diseases and regenerative medicine therapies.

2.3.3.1. Autoimmune disease therapy. Autoimmune diseases are a chronic group of diseases that arise from an inappropriate immune response against self-antigens resulting in inflammation and destruction of healthy tissues. Nanocarriers used to deliver anti-inflammatory molecules into target tissue are lipid-based NMs such as liposomes, wrapsome, gemini-lipid NPs, and micelles (Allen and Cullis, 2013), polymer-based NMs such as polyethylenimine, PLA, PLGA, or chitosan and collagen (Gharagozloo et al., 2015). Biodegradable PLGA NMs entrapping collagen II significantly suppressed rheumatoid arthritis and tumor necrosis factor (TNF α) expression in treated animals, suggesting that the slow sustained release of collagen from PLGA may provide a suitable delivery system for oral tolerance induction (Kim et al., 2002). Certolizumab

pegol (Cimzia[®]), a nanosized anti-TNF α fragment (Fab'), conjugated to a PEG moiety, manifests improved stability and prolongs half-life in circulation (Markatseli et al., 2013). Cationic polymer or lipid-based vehicles are the main delivery systems for siRNA which has manifested impressive therapeutic potential against autoimmune diseases (Khoury et al., 2006). Some NMs such as CNTs, Au, and cerium oxide have been shown to possess immunosuppression activity *per se*, along with anti-oxidative and anti-inflammatory properties (Schanen et al., 2013; Sumbayev et al., 2013; Serra and Santamaria, 2015). These NMs with desired tropism might be further engineered to preferentially accumulate in specific subcellular compartments of immune cell types. Rapamune[®], a nano-crystal form of rapamycin used for the treatment of autoimmune diseases and transplant rejection possesses higher bioavailability compared to the oral solution (Junghanns and Müller, 2008).

2.3.3.2. Cancer immunotherapy. Tumors are known to not only avoid immune surveillance but also exploit the immune system to continue local tumor growth and metastasis. Vaccination of dendritic cells, *i.e.* efficient and targeted delivery of immunomodulatory and co-stimulatory molecules to antigen-presenting cells using NMs, represents a promising strategy in the multimodal treatment for different types of cancer. The most commonly used nanocarriers are lipid- and polymer-based NMs (Shargh et al., 2016), Au and silica NMs (Almeida et al., 2014; Shahbazi et al., 2015). Immunotherapy can also be used in combination with other treatment modalities such as chemotherapy, phototherapy, and gene therapy. Co-delivery of paclitaxel and a Toll-like receptor 4 agonist through a PLGA-based NM preparation has shown better tumor regression compared with conventional administration of paclitaxel and enhancement of antitumor immune response at the tumor micro-environment *in vivo* (Roy et al., 2013).

An exciting new area of research and development is the construction of nanorobots, *i.e.* mechanical nanodevices at the nanoscale and their applications in areas such as hematology, dentistry, neurosurgery or oncology (Saadeh and Vyas, 2014).

3. Adverse health effects

By directly interacting with the genetic material or disturbing the mitotic spindle and its components, NMs can induce genotoxicity and mutagenicity (Kumar and Dhawan, 2013; Magdolenova et al., 2014). NMs can interact with biomolecules involved in normal genome function or cell division (Magdolenova et al., 2014). Many cellular signalling pathways have been shown to be involved in the underlying mechanisms of genotoxicity of NMs. Nanotoxicity is mediated also by inflammatory responses of macrophages and neutrophils (Stone et al., 2009), via production of reactive oxygen species (ROS) (Magdolenova et al., 2014; Dusinska et al., 2015). Production of ROS and reactive nitrogen species (RNS) induces inflammatory cell recruitment and activation, and thus induction of an inflammatory response in the cells. In addition to oxidative stress and inflammation, the International Agency for Research on Cancer (IARC) has highlighted biopersistence, incomplete phagocytosis, activation of oncogenes or inactivation of tumor suppressor genes as important mechanisms of action leading to carcinogenicity of genotoxic NMs (IARC Working Group, 2002, 2006). In 2014 the IARC evaluated the risk of human cancer development in relation to exposure to CNTs, in the first attempt to assess the impact of exposure to NMs on cancer risk (Grosse et al., 2014). Additionally to oxidative stress, inflammation and genotoxicity, other mechanisms such as epigenetic modifications may contribute to adverse health effects.

4. Nanomaterials can affect epigenetic regulation

Epigenetics, via DNA methylation, histone modifications and interacting regulative non-coding RNAs, allows sophisticated, time- and tissue-specific control of gene expression in both normal and pathological development (Jenuwein and Allis, 2001). Epigenetic marks are generally reversible and can be influenced by environmental factors. Although hundreds of studies have investigated environmentally induced epigenetic toxicity, relatively few have demonstrated a mechanistic link between specific environmental exposures, epigenetic changes and adverse phenotypes. Current evidence for putative environmentally induced epigenetic toxicity in human epidemiological cohorts has been reviewed recently (Marczylo et al., 2016). These data provide support for associations between various types of environmental exposure, altered epigenetic mechanisms and development of childhood asthma, behavioural abnormalities, AD, skin abnormalities, lung cancer, chromosomal aberrations and chronic obstructive pulmonary disease.

Several materials, mainly heavy metals, have been qualified as epimutagens. They were shown to alter DNA methylation and histone modifications (Cheng et al., 2012). The epigenetic toxicity of these and other materials in nanoscale has been tested, to secure their safe utilization in nanomedical applications. The first report that NMs can cause significant epigenetic toxicity was published in 2008 (Choi et al., 2008). Since then the number of publications on the epigenetic toxicity of MNs has significantly increased. However, our understanding of how NM exposure influences the epigenome and its contribution in development of diseases is far from complete. Stocco and colleagues were among the first to summarize the epigenetic effects of NM exposure (Stocco et al., 2013). They demonstrated NM-induced changes in DNA methylation, methylation and acetylation of histones, and expression of miRNAs. It was shown that certain NMs can impair the expression of genes involved in DNA methylation reactions, leading to more serious global changes. Apart from a limited number of human studies, *in vitro* and animal studies have been instrumental in increasing our understanding of NM-induced epigenetic changes.

4.1. DNA methylation

DNA methylation is one of the best-studied epigenetic mechanisms. It involves covalent addition of a methyl group ($-CH_3$) to the 5th position of the cytosine residue in the dinucleotide sequence CpG. While CpG dinucleotides in the genome are generally methylated, the promoter regions of approximately 70% of human genes contain long stretches of CpGs called CpG islands, that are predominantly unmethylated (Deaton and Bird, 2011). DNA methylation in promoters prevents binding of transcription factors to target genes. Methylation/demethylation of CpG islands is therefore an important mechanism for maintenance of cell- or tissue-specific gene expression either directly or indirectly via CpG binding proteins (MBDs). Global DNA methylation plays a role in maintaining genome integrity. Global DNA hypomethylation has been associated with increased chromosome instabilities and tumorigenesis.

Several enzymes have crucial roles in DNA methylation. In mammals, DNA methylation is mediated by three types of DNA methyltransferases (DNMTs), which either enable *de novo* DNA methylation (DNMT3A and DNMT3B) or are responsible for maintaining methylation status during cell division (DNMT1). The discovery of ten-eleven translocation (TET) proteins has shed light on the DNA demethylation mechanisms. The TET family of 5-methylcytosine (5-mC) hydroxylases have been shown to function in transcriptional activation and repression, tumour

suppression and DNA methylation reprogramming. TET proteins are capable of catalysing the oxidation of 5-mC into 5-hydroxymethylcytosine (5-hmC) in mammalian DNA (Tahiliani et al., 2009). The effect of DNA methylation is indirectly mediated through proteins that bind to symmetrically methylated CpGs. They contain a specific domain - MBD, which is linked to additional domains associated with chromatin. MBD-containing proteins have a role in recruiting a variety of histone deacetylases (HDACs) and chromatin remodelling factors. The interaction between DNA methylation and histone modifications leads to chromatin remodelling and transcription repression. The role of aberrant DNA methylation in complex and rare diseases is well documented; however, causal links between epigenetic changes induced by environmental exposure and disease phenotypes have yet to be elucidated.

4.2. Histone modifications

In comparison with DNA methylation, the most diverse epigenetic modifications are those that occur on histone proteins. In nucleosomes, DNA is wrapped around octameric proteins called histones. Nucleosome spacing determines the structure of chromatin into heterochromatin (condensed structure, transcriptionally inactive) or euchromatin (open structure, transcriptionally active). Several covalent modifications of the N-terminal histone tails protruding into the nuclear lumen, such as acetylation, methylation, phosphorylation, suomylation, ubiquitylation, ATP ribosylation and others lead to changes in chromatin structure that can influence transcription (Jenuwein and Allis, 2001). Recent genome-wide studies demonstrate that actively transcribed or transcriptionally repressed regions are characterized by a specific modification pattern. For example, di- or tri- methylation of histone 3 (H3) at lysine (K) 4 and 36 - H3K4me2, H3K4me3, H3K36me2 and H3K36me3, are frequently located in actively transcribed regions of the genes. On the contrary, H3K27me3 and H4K20me3 are frequently mapped to regions where transcription is repressed (Barth and Imhof, 2010). Histone modifications are extremely dynamic and highly regulated by a complex of histone-modifying enzymes including HDACs, histone acetyltransferases (HATs), methyltransferases (HMTs) or demethylases (HDMs). Their aberrant functions may cause misregulation of chromatin structure and activity, with an impact on access of transcription factors to DNA and gene transcription. Histone-modifying enzymes can be divided into distinct groups based on broad functions. Epigenetic writers, that lay down epigenetic marks (e.g. HATs, HMTs), are recognized by epigenetic readers, the enzymes that bind to these modifications (e.g. bromodomains, chromodomains) and are removed by epigenetic erasers (e.g. HDACs or histone lysine demethylases). In addition to DNA and histone modifications, chromatin structure and function are regulated by chromatin remodelling complexes, non-coding RNAs and mutations in histone proteins themselves (Dawson and Kouzarides, 2012). The effect of NM exposure on histone modifications has been studied to a lesser extent than DNA methylation changes. Several studies have shown that histone modifications are important molecular targets for different types of NMs. The entry of NMs into the cell nucleus can modulate different cellular functions depending on the chromatin region affected.

4.3. Non-coding RNAs

Several classes of non-coding RNAs (ncRNAs), classified as regulatory ncRNAs, are involved in the posttranscriptional regulation of gene expression. Both short ncRNAs (<30 nucleotides) as well as long ncRNAs (>200 nucleotides) are shown to play a role in DNA methylation targeting, histone modifications and heterochromatin

formation.

Short ncRNAs include three classes of RNAs that are not translated into proteins. The best-studied class comprises evolutionarily conserved, small single-stranded molecules, 20–24 nucleotides long, termed microRNAs (miRNAs). Deregulation of miRNAs has been implicated not only in tumorigenesis, but also in neurological, cardiovascular, developmental and other diseases (Esteller, 2011; Paul et al., 2017). They have diverse functions in the cells and it is estimated that more than a third of the mammalian genes are regulated by miRNAs (Kaikkonen et al., 2011). The miRNAs regulate gene expression by interfering with the mRNA processes, affecting mRNA stability, targeting mRNA for degradation, or both (Mathers et al., 2010). They also can affect DNA methylation and histone modification by regulation of DNMTs and histone modifiers. Short interfering RNAs (siRNAs) function in a very similar way. They mediate post-transcriptional gene silencing as a result of mRNA degradation; however, they have also been shown to induce heterochromatin formation via an RNA-induced transcriptional silencing (RITS) complex. Piwi-interacting RNAs (piRNAs) are small ncRNA molecules of 24–31 nucleotides in length, whose primary function involves chromatin regulation and suppression of transposon activity. In contrast to miRNAs, piRNAs interact with PIWI proteins and function primarily in the nucleus. They have been uncovered in the germline, but growing evidence supports their role in somatic cells, where they are expressed in a tissue-specific manner (Wu et al., 2010; Siddiqi and Matushansky, 2012).

Long ncRNAs (lncRNAs) are the least-understood ncRNA species. They play a wide range of regulatory function as signals or decoys by involvement in gene activation and suppression. They can influence gene expression as guides by recruitment of chromatin-modifying proteins catalytic activity to specific sites in the genome. They function as scaffolds in chromatin remodelling or modulate histone marks (Chen et al., 2017). They can regulate gene expression through their interaction with other epigenetic processes, mainly the expression of histone modifier and chromatin remodelling proteins and DNA methylation. Thanks to this plasticity and capacity for dynamic interactions, epigenetic processes mediate cell response to diverse external or internal stimuli (Zhao et al., 2016).

4.4. Epigenetic changes induced by NMs *in vitro* and *in vivo*

The metal particles constitute an important class of NMs. Traditionally, the toxicity of metals was thought to be mediated by DNA damage. Recent research has shown that exposure to metals can cause persistent changes in the epigenome. Although the epigenetic targets of heavy metals have been repeatedly reviewed (Arita and Costa, 2009; Fragou et al., 2011; Martinez-Zamudio and Ha, 2011; Cheng et al., 2012) the epigenetic effects of these substances at the nanoscale have only rarely been studied. In comparison to inorganic NMs, polymer nanocarriers (or other biodegradable devices) have been studied to an even lesser extent, although they are the most promising for pharmaceutical applications. The main reason explaining the lack of interest is the fact that materials used to make polymer NMs are considered as safe. However, the properties and behaviour of a material at the nanoscale may differ from those observed in the macroscopic range. Specific importance should be devoted to the toxicity of the carriers, considering that in the majority of applications their purpose is the reduction of toxicity. The main findings of *in vitro* and *in vivo* studies, analysing epigenetic effects of NM exposure on various cell types and tissues, are summarized in Table 2 and Table 3.

4.4.1. Metallic nanomaterials

4.4.1.1. Gold. Gold NPs (AuNPs) have attracted wide attention in

various biomedical applications because they were considered relatively inert and biocompatible (Shukla et al., 2005; Khan et al., 2007). However, recent studies have raised concerns about their possible epigenetic toxicity. Changes in DNA methylation after AuNP exposure were reported both *in vitro* and *in vivo*. Single intratracheal administration of AuNPs for 48 h to BALB/c mice induced hypermethylation and hypomethylation of several genes (*ATM*, *CDK*, *GSR*, *GPX*) in mouse lung tissues (Tabish et al., 2017). Products of those genes are involved in double strand DNA damage sensing, cell cycle control and modulation of transcription in response to several extra- and intracellular cues, while *GPX* is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cells (Lee and Paull, 2007; Deponce, 2013; Malumbres, 2014). Exposure to antioxidant-based chiral AuNPs capped with GSH enantiomers L-GSH decreased the catalytic activities of TET proteins followed by a global decline of 5-hmC levels in human gastric cancer MGC-803 and human embryonic kidney HEK 293FT cells. Down-regulation of the mRNA expression level of *TET1* and *TET2* genes, and induced aggregation of TET proteins through oxidation of their catalytic domains led to changes affecting cell adhesion, migration, proliferation, differentiation and apoptosis (Ma et al., 2016a). Potential involvement of AuNPs in chromatin reorganization were shown by Mazumder and colleagues, who demonstrated modulation of heterochromatin connections with lamin proteins and core histones after incorporation of AuNPs into HeLa cells (Mazumder and Shivashankar, 2007). AuNPs were also found to decrease HDAC activity by binding to sulfhydryl groups on the surface of HDAC8 (Sule et al., 2008). A single intravenous application of AuNPs induced up- and down-regulation of 21 miRNAs both 1 week and 2 months post injection in the peripheral blood of Wistar rats. Of these, rno-miR-298 was found to control the expression of the *BACE1* gene, coding for a β -amyloid precursor protein involved in AD pathogenesis (Chew et al., 2012). In another study, up-regulation of miR-155 was observed concomitant with down-regulation of the *PROS1* gene that codes for S protein, a cofactor in processes controlling blood clotting (Ng et al., 2011). Balansky and colleagues reported significant AuNP-induced changes in miRNA expression and transplacental size-dependent clastogenic and epigenetic effects in the mouse fetus. Among 28 up-regulated miRNAs in fetal lung and 8 up-regulated miRNAs in fetal liver, Let-7a and miR-183 were significantly up-regulated in both organs (Balansky et al., 2013).

4.4.1.2. Silver. Silver NPs (AgNPs) are being used in a variety of applications as antimicrobial substances (dental plaque biofilms, treatment of infected wounds, catheters dressings). AgNP exposure stimulated changes in DNA methylation: augmentation of 5-mC, DNMT1, DNMT2, DNMT3a and DNMT3b levels was observed in hippocampal HT22 mouse neuronal cells (Mytych et al., 2016). Maternal exposure to AgNPs induced up-regulation of *ZAC1* gene expression accompanied by hypomethylation of *ZAC1* promoter in placental tissues. *ZAC1* is an imprinted gene, critical in the control of embryonic growth (Zhang et al., 2015). Sublethal AgNP concentrations induced a reduction in haemoglobin levels in mouse erythroleukemia cells through diminished H3K4me3 and H3K79me1 methylation, attributed to the inhibition of specific HMTs DOT-1L and MLL or to direct binding of AgNPs to histones H3/H4 (Qian et al., 2015). In cells exposed to AgNPs, expression of hsa-miR-219-5p was negatively correlated with the expression of *MTIF* and *TRIB3* genes, involved in oxidative stress, cell cycle and apoptosis (Eom et al., 2014). On the other hand, AuNPs were shown to induce enhanced mineralization in MC3T3-E1 bone cells via altered expression of specific microRNAs and their targets associated with bone formation (Mahmood et al., 2011).

4.4.1.3. Cadmium telluride quantum dots. Cadmium compounds are classified as human carcinogens that likely act via epigenetic mechanisms (Takiguchi et al., 2003; Arita and Costa, 2009; Cheng et al., 2012). However, cadmium telluride QDs (CdTe-QDs) have shown great potential for use as fluorescent tags in therapeutic targeting and in medical and molecular imaging. The first report suggesting epigenetic changes induced by CdTe-QDs was published in 2005. Lovric and colleagues showed that QD-induced cytotoxicity is characterized by chromatin condensation and membrane

blebbing (Lovric et al., 2005). Binding of CdTe-QDs to core histones and stimulation of aggregate formation was confirmed by Conroy and colleagues (Conroy et al., 2008). The incubation of human breast cancer MCF-7 cells with CdTe-QDs for 4 h or 24 h resulted in global histone H3 hypoacetylation and reduction of gene expression. Simultaneously CdTe-QD treatment increased the expression of some apoptotic genes through the activation of p53 (Choi et al., 2008). CdTe-QD exposure induced apoptosis-like cell death, accompanied by significant expression changes in the number of

Table 2
In vitro and in vivo findings for the epigenetic changes induced by metallic NM exposure.

	Nanoparticle (NP)	Cells/substrate/animals	Exposure/time	Effect of NMs exposure	Method	Reference
Gold	Citrate-coated colloidal AuNPs	Male BALB/c mice (~20 g, 7 weeks old)	Single intratracheal administration/48 h	Hypermethylation (<i>ATM</i> , <i>CDK</i> and <i>GSR</i>) and hypomethylation (<i>GPX</i>) of several genes in mouse lung tissues	LC-MS, pyrosequencing	(Tabish et al., 2017)
	Antioxidant-based chiral AuNPs capped with GSH enantiomers L-GSH 5 nm Au particles	Human gastric cancer MGC-803 and human embryonic kidney HEK 293FT cells HeLa cells	24 h incubation 1 h	Downregulation of gene expression of TET proteins followed by global decline of 5-hmC levels Modulation of heterochromatin connections with lamin proteins and core histones	immunoprecipitation fluorescent microscopy	(Ma et al., 2016b) (Mazumder and Shivashankar, 2007)
	Au colloid solution 10 nm	Recombinant human HDAC8		AuNPs were found to decrease histone deacetylase activity by binding to sulfhydryl groups on the surface of HDAC8	spectrophotometry	(Sule et al., 2008)
	Citrate-coated AuNPs	Male wistar rats 1 week and 2 months	single tail-vein injection	Deregulation of 21 miRNAs both after 1 week and 2 months	microarray	(Chew et al., 2012)
	Citrate-coated colloidal AuNPs 100 nm	MRC5 (lung fibroblast cell line) Adult male and female Swiss albino mice (strain H)	48 h or 72 h transplacental treatment on days 10,12,14 and 17 of gestation	Up-regulation of miR-155 with downregulation of <i>PROS1</i> gene, chromatin condensation Dysregulation of 28 miRNA in fetus lung, 5 up-regulated in liver, let-7 and miR-183 up-regulated in both	microarray, bisulfide sequencing microarray	(Ng et al., 2011) (Balansky et al., 2013)
Silver	AgNPs <100 nm	HT22 mouse hippocampal neuronal cell line	48 h, measurement 96 h after AgNP removal	Increased levels of 5-mC, DNMT1, DNMT3a and DNMT3b	MethylFlash™ MethylatedDNA Quantification Kit, EpiQuik™ DNMT1/DNMT3a/DNMT3b Assay Kit	(Mytych et al., 2016)
	AgNPs 8 nm synthesised by biological method	8-week-old ICR (imprinting control region) mice	intravenous infusion, 1 mg/kg at 6.5 day postcoitum	Maternal exposure decreased DNA methylation of <i>ZAC1</i> gene promoter in placental tissues	pyrosequencing and bisulfide sequencing	(Zhang et al., 2015)
	AgNPs with polyvinylpyrrolidone (PVP)-coating, 25 nm AgNPs size <100 nm	Mouse erythroleukemia (MEL) cells Human Jurkat T cell, Jurkat clone E6-1	72 h 24 h	Decreased HMT DOT-1L and MLL levels and direct binding of AgNPs to H3/H4 63 altered miRNAs, hsa-miR-219-5p negatively correlated with the expression of <i>MTIF</i> and <i>TRIB3</i> genes	ChIP-PCR microarray	(Qian et al., 2015) (Eom et al., 2014)
	AgNPs 23 nm prepared by borohydrate mediated reduction of silver nitrate	MC3T3-E1 bone cells	24 h	Altered miRNA expression resulting in specific gene expression associated with bone formation	RT-PCR pathway specific array	(Mahmood et al., 2011)
Quantum dots	Anionic cadmium telluride QDs (CdTe-QDs) 2.2 nm and 5.2 nm	Rat pheochromocytoma cells (PC12) and N9 murine microglial cells	24 h	Chromatin condensation and membrane blebbing	confocal microscopy	(Lovric et al., 2005)
	Anionic CdTe-QDs 3.4 nm	THP1 (human acute monocytic leukemia)	4 h or 24 h	Binding to core histones and stimulation of aggregate formation	dot blot assay	(Conroy et al., 2008)
	CdTe-QDs	MCF-7 (human breast adenocarcinoma cells)	4 h or 24 h	Histone 3 deacetylation and chromatin decondensation	fluorescence-based imaging	(Choi et al., 2008)
	CdTe-QDs 1–2.5 nm	Mouse NIH/3T3 fibroblasts	12 h and 24 h	Expression of 51 miRNAs significantly affected, resulting in the apoptosis-like cell death	SOLiD sequencing-based microRNA expression profiling	(Li et al., 2011b)
CdTe-QDs	Mouse NIH/3T3 fibroblasts	12 h and 24 h	Global alteration of miRNAs expression patterns resulting in the apoptosis-like cell death	SOLiD sequencing-based microRNA expression profiling	(Li et al., 2011a; Sun et al., 2013)	

(continued on next page)

Table 2 (continued)

	Nanoparticle (NP)	Cells/substrate/animals	Exposure/time	Effect of NMs exposure	Method	Reference
Titanium	anatase TiO ₂ NPs 22.1 nm	A549 lung cells	24 h	Hypermethylation of the <i>PARP1</i> promoter	methylation-specific PCR	(Bai et al., 2015)
	TiO ₂ NPs 21 nm	Human and murine macrophages (THP-1 and RAW264.7) human small airway epithelial cells (SAEC)	24 h	Increased methylation level of SINE1; increased expression of TET2	HPLC-ESI (5-mC; 5-hmC); methylation-sensitive qRT-PCR (LINE-1, Alu/Sine)	(Lu et al., 2016)
	TiO ₂ NPs <100 nm	Lung fibroblast MRC5 cells	24 h and 48 h	DNA hypomethylation, decrease in DNMT activity and downregulation of endogenous DNMT1, 3A and 3B expression levels	enzyme-linked immunosorbent assay-based kit	(Patil et al., 2016)
		HaCaT cells		Dysfunction of methylation cycle and methionine deficiency		(Tucci et al., 2013)
		C57BL/68om Tac female mice		Up-regulation of miR-449, miR-1 and miR-135b, targeting genes involved in inflammation and immune response in lung	microarray, validation by RT-PCR	(Halappanavar et al., 2011)
	TiO ₂ NPs 38 nm	A549 lung cells	24 h	Persistent down-regulation of miR-21 and miR-30a, changes in miR-155 expression	qRT-PCR	(Alinovi et al., 2017)
Iron	SPIOs, 4–7 nm	PC12 rat pheochromocytoma, neuroendocrine cell line	24 h	Widely changed miRNA expression pattern; triggering neuron degeneration pathways	SOLiD sequencing	(Sun et al., 2015)
	Fe ₂ O ₃	Mouse NIH/3T3 fibroblasts	12 h and 24 h	Genome wide changes in miRNAs expression, number of KEGG pathways and GO terms affected	SOLiD sequencing-based microRNA expression profiling	(Li et al., 2011a)
Zinc	ZnO 90 nm	Human embryonic kidney HEK-293 cells	48 h	Global reduction in 5-mC and increase in 5-hmC content; increase in the expression of <i>TET1</i> and <i>TET2</i> genes	MethylFlash Quantification Kit (colorimetric assay)	(Choudhury et al., 2017)
	ZnO <100 nm	Lung fibroblast MRC5 cells	24 h and 48 h	DNA hypomethylation accompanied by decrease in DNMTs activity and downregulation of endogenous DNMT1 and 3A expression levels	enzyme-linked immunosorbent assay-based kit	(Patil et al., 2016)
	ZnO <100 nm	Human epidermal keratinocytes HaCaT cells	24 h	Increased methylation of H3K9 and decreased acetylation of H4K5, chromatin condensation, up-regulation of HMT G9a and downregulation of HATs GCN5, P300 and CBP	western blot and immunostaining analysis	(Gao et al., 2016)
Copper	CuO 58.7 nm	Human and murine macrophages (THP-1 and RAW264.7) human small airway epithelial cells (SAEC)	24 h	Hypermethylation of L1 and Alu, reactivation of both ORF1 and ORF2 as well as SINE B1 and SINE B2 in RAW264.7; changes in gene expression of <i>DNMT1</i> and <i>TET3</i>	methylation-sensitive quantitative RT-PCR	(Lu et al., 2016)
Cobalt	Co ₃ O ₄ NPs	Lung cells (A549)	24 h	Changes in miR-21, miR-30a and miR-155 expression; recovery at 24 h for miRNA-21 and an up-regulation for miRNA-30a	qRT-PCR	(Alinovi et al., 2017)

Alu, primate-specific repeats; DNMT1, DNA methyltransferase 1; GSH, glutathion; ORF, open reading frame; GO, gene ontology database; HAT, histone acetyltransferase; HMTs, histone methyltransferases; HPLC – ESI, high-performance liquid chromatography-electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; LINE-1, long interspersed elements; KEGG, Kyoto Encyclopedia of Genes and Genomes; qRT-PCR, quantitative real-time PCR; SINE, short interspersed elements; SPION, superparamagnetic iron oxide nanoparticles; TET, ten-eleven translocation methylcytosine dioxygenase; 5-hmC, 5-hydroxymethylcytosine; 5-mC, 5-methylcytosine.

miRNAs, in NIH/3T murine fibroblasts (Li et al., 2011a, 2011b; Sun et al., 2013).

4.4.1.4. Titanium. Titanium is extensively used for implanted medical devices, such as dental implants, joint replacements, cardiovascular stents, and spinal fixation (Bai et al., 2015). Titanium dioxide NPs (TiO₂NPs) were evaluated by the IARC as a Group 2B (possibly carcinogenic) compound (Baan, 2007). The European Chemical Agency's Committee for Risk Assessment in June 2017 concluded that the available scientific evidence meets the criteria to classify titanium dioxide including in nano-form as a substance suspected of causing cancer through inhalation (<https://echa.europa.eu/-/titanium-dioxide-proposed-to-be-classified-as-suspected-of-causing-cancer-when-inhaled>). Exposure with TiO₂NPs induced hypermethylation of *PARP1* promoter, triggered by oxidative stress (Bai et al., 2015). PARP-1 is a highly conserved DNA-binding protein involved in many molecular and cellular processes, including DNA repair, proliferation, and chromatin modification (Chevanne et al., 2007). Short-term exposure to 21 nm TiO₂NPs induced a modest increase in DNA methylation of SINE

repetitive sequences, their transcriptional reactivation, and elevation of expression of *TET2* in the RAW264.7 murine cell line. However, the global level of 5-mC and 5-hmC remained unchanged (Lu et al., 2016). A decrease in global DNA methylation accompanied by a decrease in DNMT activity and downregulation of endogenous *DNMT1*, 3A and 3B expression levels were shown in MRC5 cells on 24 and 48 h exposure to TiO₂NPs (Patil et al., 2016). Different crystal phases of TiO₂NPs (anatase, rutile and anatase: rutile mixture; 20–26 nm) were studied for cytotoxicity, genotoxicity and global DNA methylation and hydroxymethylation in bronchial epithelial 16 HBE cells. Epigenetic modifications were found to occur already at sub-cytotoxic and sub-genotoxic concentrations (Ghosh et al., 2017).

Treatment of HaCaT cells with TiO₂NPs caused dysfunction of the methylation cycle and methionine deficiency (Tucci et al., 2013). As the methionine cycle has a crucial role in the availability of methyl groups for methylation processes in living cells, deregulation of the cycle can have a dramatic impact on global as well as gene-specific DNA methylation, particularly during embryogenesis. In lungs of mice exposed by TiO₂NPs, significantly

Table 3
In vitro and *in vivo* findings for the epigenetic changes induced by non-metallic NM exposure.

	Nanoparticle (NP)	Cells/substrate/animals	Exposure/time	Effect of NP exposure	Method	Reference
Silica	15 nm SiO ₂	Immortalized epithelial HaCaT cell line	24 h	Global hypomethylation; dose-dependent decrease in <i>DNMT1</i> , <i>DNMT3a</i> and <i>MBD2</i> gene and protein expressions	flow cytometry assay, HPCE	(Gong et al., 2010)
		HaCaT cell line		<i>PARP-1</i> hypermethylation and repression of gene expression		(Gong et al., 2010)
	70 nm	BEAS-2B cells exposure over 30 passages		DMRs in promoters of 32 genes; DNA hypermethylation of <i>CREB3L1</i> and <i>BCL2</i>	HumanMethylation450 BeadChip	(Zou et al., 2016)
		BALB/c mice	6–7 weeks old, 4, 8, 24, or 72 h after treatment	Up-regulation of miR-122	TaqMan RT-PCR	(Nagano et al., 2013)
Carbon nanotubes	C60, MWCNTs	A549 lung cells	24 h	Elevation of global DNA methylation level	HPLC-MS	(Li et al., 2016)
	SWCNTs	Male BALB/c mice (~20 g, 7 weeks old)	Single intratracheal administration, 48 h	Slight promoter hypomethylation in <i>ATM</i> gene	LC-MS, bisulfite pyrosequencing	(Tabish et al., 2017)
	MWCNTs	C57BL/6 mice oropharyngeal instillation, lung and blood tissues	Acute (24 h post-exposure) and subchronic exposures (7 days post-exposure)	Hypomethylation of <i>IFNG</i> , <i>TNF</i> and hypermethylation of <i>THY1</i> in lung, global hypomethylation in both lung and blood	LUMA and 5 mC quantification assay, pyrosequencing	(Brown et al., 2016)
	MWCNTs	Mouse NIH/3T3 fibroblasts	12 h and 24 h	Changes of various miRNAs expression, only three KEGG pathways were significantly regulated	SOLiD sequencing-based microRNA expression profiling	(Li et al., 2011a)
Hydroxyapatite	Nano-hydroxyapatite, 100 × 10 nm	Mouse bone Marrow stromal cells and MC3T3-E1 (pre-osteoblasts) and MLO-Y4 (osteocytes)		Promoter Hypomethylation of <i>ALP</i> , <i>BSP</i> and <i>OSC</i> genes		(Ha et al., 2015)
Scaffolds	Polydimethylsiloxane surface or aligned poly(L-lactide-co-caprolactone) nanofibers Nanofibrous scaffolds			Histone modifications		(Xu et al., 2013b)
				Decreased HDAC activity, increased acetylation and methylation of H3		(Downing et al., 2013)
Soft NMs	Cholesterylbutyrate solid lipid NPs releasing butyric acid, 100–150 nm K-182 HDACi-coated cationic NPs			HDAC inhibition		(Brioschi et al., 2008)
				Increase in gene expression and core histone hyperacetylation		(Ishii et al., 2009)

DMRs, differentially methylated regions; DNMT, DNA methyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HPCE, high performance capillary electrophoresis assay; HPLC-MS, high performance liquid chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LUMA, luminometric methylation assay; MBD2, methyl-CpG-binding domain protein 2.

altered expression of several miRNAs was detected, including miR-449, miR-1 and miR-135b, targeting genes involved in inflammation and immune response (Halappanavar et al., 2011). TiO₂NPs exposure induced persistent (lasting for 48 h) down-regulation of miRNA-21 and miRNA-30a involved in the autophagy pathway. Up-regulation of miR-155 was observed after 2 h, with a subsequent decrease at longer times of exposure (Alinovi et al., 2017).

4.4.1.5. Iron. SPIONs are widely accepted as powerful MRI diagnostic agents as well as drug carriers targeting CNS. They were rarely studied from the view of epigenetic toxicity; however, they caused wild changes in the miRNA expression pattern after 24-h treatment of PC12 rat pheochromocytoma neuroendocrine cells. The bioinformatic tools Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment identified changes in genes related to cell death or apoptosis, indicating that SPIONs may trigger neuron degeneration pathways (Sun et al., 2015). 24-hour exposure by iron oxide NPs (Fe₂O₃NPs) induced changes in gene expression of 167 miRNAs in NIH/3T3 cells. Several

KEGG pathways and GO terms were significantly affected, including the lipid biosynthetic process and cellular lipid metabolism (Li et al., 2011a).

4.4.1.6. Zinc. The application of zinc oxide NPs (ZnONPs) with a size of 100 nm is generally recognized as safe by FDA, due to their better biocompatibility compared with smaller ZnONPs (Rasmussen et al., 2010). Recently they were shown to induce epigenetic modifications in human embryonic HEK-293 kidney cells (Choudhury et al., 2017). The authors determined the global and locus-specific changes in DNA methylation at three genomic repeat sequences, namely global LINE-1, subtelomeric D4Z4 and pericentromeric NBL2, and at the promoters of selected ROS responsive genes (*AOX1*, *HMOX1*, *NCF2*, *SOD3*). They found a global reduction in 5-mC and increase in 5-hmC content accompanied by a significant increase in the expression of TET but not DNMT enzymes. This finding strengthens the possibility of proactive TET activity inducing hydroxymethylation marks in ZnONP treated cells, thus causing genome-wide hypomethylation (Choudhury et al., 2017). Patil and

colleagues showed a dose-related decrease in global DNA methylation and DNMT activity on 24- and 48-h treatment of lung fibroblast MRC5 cells with ZnONPs. They also found a direct correlation between the concentration of particles, global methylation levels, and expression levels of *DNMT1*, *3A* and *3B* genes on exposure (Patil et al., 2016). ZnONPs <100 nm also induced chromatin changes and condensation in human epidermal keratinocytes after 24 h, including increased methylation of histone H3K9 and decreased acetylation of histone H4K5 (Gao et al., 2016). These epigenetic changes were accompanied by decreases of gene expression of HAT genes *GCN5*, *P300* and *CBP* and simultaneous increases in HMT genes *G9* and *GLP* as well as apoptotic genes *BAX*, *NOXA* and *PUMA*. These findings suggest that the ZnONPs induced the cell cycle arrest at G2/M that is often linked to the DNA damage response and involves changes in histone modifications (Groth et al., 2007).

4.4.1.7. Copper. Copper oxide NPs (CuONPs) are used as antimicrobial reagents and for manufacturing intrauterine contraceptive devices (Xu et al., 2013a). Lu and colleagues exposed human and murine macrophages (THP-1 and RAW264.7, respectively) and human small airway epithelial cells (SAEC) to CuONPs. Their effects on the cellular epigenome were determined by addressing the global (5-mC and 5-hmC levels) and transposable element-associated DNA methylation (LINE-1 and *Alu*/SINE DNA methylation) and gene expression. They showed that exposure to CuONPs slightly decreased LINE-1 methylation in RAW264.7 mouse macrophages while an increase in DNA methylation of *Alu* and LINE-1 sequences was detected in human THP-1 and SAEC cell lines. The decrease in LINE-1 methylation was followed by enhanced transcription of *Alu-1* and SINE repetitive elements in RAW264.7 mouse macrophages. Exposure to CuONPs was accompanied by suppression of *TET1*, *TET2* and *TET3* expression (Lu et al., 2016).

4.4.1.8. Cobalt. Medical applications of cobalt in the form of cobalt ferrite NPs include hyperthermia therapy, MRI, magnetic separation and drug delivery (Amiri and Shokrollahi, 2013). Although not yet studied for the ability to cause epigenetic changes, cobalt oxide NPs (Co₃O₄NP), mostly used in industrial applications, induced temporary changes in miR-21 and miR-30a expression. Increase in miR-155 expression was observed after 2 h with a subsequent decrease at longer times of exposure (Alinovi et al., 2017). Selection of miRNA for the analysis was focused on the autophagy pathway that can be crucial in tumorigenesis. However, the oxidative stress caused by Co₃O₄NPs hampered the ability to detoxify and to repair the resulting damage, thus preventing the induction of autophagy.

4.4.2. Non-metallic nanomaterials

4.4.2.1. Silica. Nanosized silica (SiNPs) has a great potential for a variety of diagnostic and therapeutic applications in medicine, such as cancer therapy, DNA transfection, drug delivery, and enzyme immobilization (Napierska et al., 2010). SiNPs were found to induce global hypomethylation *in vitro*, associated with a dose-dependent decrease in DNMT1, DNMT3A and MBD2 gene and protein expression as well as global histone hypoacetylation, implying a global epigenomic response (Gong et al., 2010). The same group also discovered a decrease in the gene expression of the pivotal DNA repair enzyme PARP-1 induced by SiNP exposure (Gong et al., 2012). PARP-1 detects and relocates to single strand breaks and modifies nuclear proteins including histones by relaxation of chromatin, thus facilitating the access of repair proteins to damaged DNA. The low-dose SiNP exposure was evaluated in human bronchial epithelial BEAS-2B cells over 30 passages. The authors showed that long-term low-dose exposure to SiNPs caused changes in DNA methylation. They identified 32 differentially methylated regions including hypermethylation of *CREB3L1* and

BCL2 genes, associated with mitochondrial-mediated apoptosis via the PI3K/Akt signalling pathway (Zou et al., 2016). Nagano and colleagues reported changes in miR-122 expression as a biomarker of liver damage in mice, induced by exposure to SiNPs of 70 nm diameter (Nagano et al., 2013).

4.4.2.2. Carbon nanotubes. Low-dose carbon-based fullerene NMs (C60) and multi-walled CNTs (MWCNTs) significantly elevated global DNA methylation in human lung epithelial A549 cells after 24-h incubation (Li et al., 2016). Single-walled CNTs (SWCNTs) induced also a slight decrease of DNA methylation in the promoter of the *ATM* gene in lungs. Surprisingly, more genes were epigenetically altered with AuNPs than with CNTs, in contrast with the paradigm that exposure to AuNPs does not induce an adverse biological response (Tabish et al., 2017). Alterations in DNA methylation, corresponding with lung inflammation, induced by the exposure to MWCNTs in C57BL/6 mice were shown by another group (Brown et al., 2016). In this study, promoter methylation of inflammatory genes (*IFNG* and *TNF*) correlated with initial cytokine production. DNA methylation of a gene involved in tissue fibrosis (*THY1*) was also altered in a way that matched collagen deposition. In addition, MWCNT exposure led to DNA hypomethylation in the lung and blood, which coincided with disease development. MWCNT exposure was also shown to induce changes in expression of 175 miRNA in NM treated NIH/3T3 cells. Only three KEGG pathways were significantly regulated by miRNAs in MWCNT treated cells, suggesting the good biocompatibility of MWCNTs when compared to Fe₂O₃NPs and CdTe-QDs (Li et al., 2011a).

4.4.2.3. Hydroxyapatite. HA is the primary structural component of the skeleton and dentition. It is currently being investigated as a promising therapeutic biomaterial for use as a functional scaffold and implant coating, for skeletal repair and dental applications. A biological effect of HANPs (10 × 100 nm) on the lineage commitment and differentiation of bone-forming osteoblasts was studied recently (Ha et al., 2015). Exposure of early stage differentiating osteoblasts to HANPs resulted in dramatic and sustained changes in gene expression and stimulation of DNA methylation of the alkaline phosphatase gene (*ALPL*).

4.4.2.4. Soft nanomaterials. It was hypothesised that the shape, or more precisely the topography of NMs used as scaffolds for biological and medical applications, seems to affect the cellular epigenome, specifically through histone modification alterations (Sierra et al., 2016). This hypothesis was supported by the finding that reprogramming of somatic cells as pluripotent stem cells is more efficient when bioengineered substrates are used. The micro- and nano-patterned polydimethylsiloxane surface or aligned poly(L-lactide-co-caprolactone) nanofibers significantly promoted the expression of epithelial and pluripotency markers by triggering a more elongated cell morphology, which led to the induction of histone modifications. This process is thought to be mediated by mechanotransduction signalling through the cytoskeleton (Xu et al., 2013b). The mechanomodulation of the cells' epigenetic state was shown also for nanofibrous scaffolds with aligned fibre orientation that promoted a mesenchymal-to-epithelial transition in adult fibroblasts. Decreased HDAC activity and up-regulation of the expression of WDR5, a subunit of H3 methyltransferase, by microgrooved surfaces led to increased histone H3 acetylation and methylation. These findings can have important implications in cell biology and nanomedicine in the optimization of biomaterials for cell-engineering applications (Downing et al., 2013). Solid lipid NPs (SLNs), whose lipid matrix is cholesterylbutyrate (Chol-but) could be regarded as suitable and highly effective pro-drug of butyric acid. Butyrate is regarded as an endogenous member of the family

of HDAC inhibitors. However, similarly to other non-selective HDACi, Chol-but SLNs seems to act by modulating different pathways in a non-specific manner. The pathways were mainly involved in cell survival and proliferation, which can represent a severe limitation because of undesired and unavoidable local and systemic effects (Brioschi et al., 2008). K-182 HDACi-coated cationic NMs were prepared as a DNA vector to transfect plasmid DNA into human prostate cancer cells, PC-3, or human breast cancer cells, Sk-BR-3. They resulted in an increase in gene expression and core histone hyperacetylation (Ishii et al., 2009).

4.5. Implications for human health

Despite the international effort focused on the identification of normal tissue-specific variability of the epigenome (International Human Epigenome Consortium, <http://ihec-epigenomes.net>), the causal links between epigenetic changes and complex diseases remain elusive. Decreased availability of methyl donors, direct changes in epigenetic marks or abnormalities of the epigenetic modifiers can contribute to disease pathogenesis. Epigenetic regulation plays crucial roles mainly during early development in embryogenesis, controlling cell differentiation. Environmental exposure in this most sensitive period can cause mitotically inheritable changes in the epigenome that pass to the next generation, thus modifying disease susceptibility. The finding that environmental factors such as diet or exposure can alter the epigenome provides an argument that epigenetic factors, as functional modifiers of the genome, are key determinants of disease risk. Besides inherited disorders, many common complex diseases such as cancer, schizophrenia, autism, diabetes and others have been associated with epigenetic alterations induced by environmental exposure.

Global hypomethylation in tumors was one of the first epigenetic alterations associated with human cancers. Changes in global DNA methylation may induce a number of adverse outcomes, including chromosome instability, activation of intragenomic endoparasitic DNA such as L1 and Alu repeats, and loss of imprinting (Berdasco and Esteller, 2010). Hypermethylation in promoter regions of tumor-suppressor genes, affecting a range of cellular functions such as cell cycle, DNA repair, metabolism, apoptosis and many others is accepted as a common feature of tumorigenesis (Esteller, 2008). Both global as well as gene-specific DNA methylation aberrations were shown to be induced by NM exposure as shown above. The causal role of epigenetic changes in cancer has been proved by the identification of driver mutations in epigenetic regulators that are highly recurrent somatic alterations across a wide range of cancer types. Compilation of the epigenetic regulators mutated in cancer, highlights histone acetylation and methylation as the most widely affected epigenetic pathways (Dawson and Kouzarides, 2012). However, the great diversity in histone modifications and their dynamic changes introduces a remarkable complexity that is only slowly beginning to be elucidated. The role of non-coding RNAs in normal development and disease is also increasingly recognized. Because their expression patterns often correlate with a disease type or subtype, they can provide specific diagnostic or even prognostic clinical information. Bearing in mind the capacity of epigenetic changes for 'resetting', there are several examples of how epigenetically deregulated tissue-specific genes in cancer could restore their differentiated phenotype (Berdasco and Esteller, 2010). Understanding the exact epigenetic mechanism governing carcinogenesis and other pathologies can have significant therapeutic consequences.

Beyond cancer, several neurodevelopmental disorders have been associated with changes in epigenetic mechanisms (Mohan and Chaillet, 2012). Taking into account the central role played by

the epigenetic machinery in development, their number is surprisingly low. However, the majority of these changes are not viable, due to their large-scale effects during embryogenesis. Albright's hereditary osteodystrophy, Angelman, Beckwith–Wiedemann, and Prader–Willi syndromes comprise a group of diseases whose aetiologies include deregulation of imprinted genes. Aberrant methylation of repeated sequences or gene-specific sequences is involved in the aetiology of alpha thalassemia X-linked intellectual disability, Fragile X, and the immunodeficiency, centromeric region instability and facial anomalies (ICF) syndromes. Mutations of epigenetic modifiers are involved in ICF (*DNMT3b*), Rett (*MeCP2*), Rubinstein-Taybi (*CREBBP*) or Coffin-Lowry (*RSK-2*) syndromes. Autism, one of the most common types of neurobiological disorders, is now thought to be caused not only by mutations of several synapse-related genes, but also by their acquired epigenetic dysregulation, induced by environmental factors (Kubota et al., 2012).

Disrupted CpG methylation status of a set of common genes was shown for a group of several neurodegenerative disorders, e.g. AD, dementia with Lewy bodies, PD and AD-like neurodegenerative profile associated with Down's syndrome. This finding is particularly important, suggesting that neurodegenerative disorders might have similar early pathogenic mechanisms that subsequently evolve into clinical entities with different molecular and cellular features (Sanchez-Mut et al., 2016). Although the aetiology of sporadic AD is still uncertain, environmental factors such as diet and exposure to heavy metals were shown to be involved, specifically in its late-onset form. Abundant evidence has advocated that development and course of AD are associated with epigenetic mechanisms (Qazi et al., 2017).

A strong association has been discovered between environmental factors, age and development of autoimmune disorders. Epigenetic factors are mediators between environment and disease and it is thought that they can be responsible for the development and progression of systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, psoriasis, Crohn's disease and several other inflammatory and chronic autoimmune disorders (Javierre et al., 2012). Early environmental factors appear to be a critical component also for the development of numerous allergic diseases (Amarasekera et al., 2012).

Mounting evidence has indicated that epigenetic alterations have critical functions in modulating smooth muscle and endothelial cell homeostasis, thus supporting their function in cardiovascular diseases (Baccarelli and Ghosh, 2012). Environmentally induced changes in epigenetic signatures were reported also for obesity and type 2 diabetes (Ling and Groop, 2009; Van Dijk et al., 2015).

The reversible character of epigenetic regulations and their responsiveness to external stimuli give us the opportunities for prevention, delay or treatment of complex disorders. Elucidation of the causal link between exposure-induced epigenetic aberrations and human diseases is an inevitable step for successful application of epigenetic-based therapies and risk assessment.

5. Future perspectives

Despite the growing body of evidence that support a real risk resulting from NM exposure for human health, this topic remains controversial especially in the area of nanomedicine.

Although global DNA methylation changes were shown in response to NM exposure, the majority of reports do not provide correlation with gene expression, which makes it difficult to interpret their possible functional effects. On the other hand, global DNA methylation changes may be masked by the redistribution of methylation patterns between the different genomic loci, where

the hypomethylation of one and hypermethylation of others may result in cumulatively unchanged levels of global methylation (Miousse et al., 2014). Several studies have identified methylation changes in response to NM exposure at specific loci. It should be noted that, generally, promoter hypermethylation is associated with gene silencing, whereas the effect of intragenic methylation is not so clear, although it might also have a role in regulating gene expression. Discrepancies in microarray-based findings of individual studies can be in part explained by differences in treatment conditions, NM-properties (coating, size etc.) and individual microarray platform probe design. NM-induced changes in chromatin reorganization and altered HDAC and TET activities, implying global epigenomic response, have been repeatedly reported. Many miRNAs were co-regulated after two out of three NM exposures which suggests the similarity of epigenetic effects for several NMs. Due to the small number of studies, overlap between *in vivo* and *in vitro* studies is often missing. Despite the variation in published data and challenges that epigenomic research faces, the association between NM exposure and epigenetic changes remains biologically plausible. There is a need to distinguish between the physiological response of the cells to the exposure and pathological changes. Further investigations into the long-term effects of NMs on epigenetic modifications and subsequent recovery assays after NP exposure will be required. Epigenetic effects of coated and soft carriers, that are usually used to prevent toxicity, should be studied.

The data from human epidemiological studies, showing direct effects of NM exposure on disease risk, are lacking. The evidence for epigenetic toxicity of NMs is mostly based upon *in vitro* and animal models. It may be extremely problematical to transpose findings made under controlled laboratory conditions to the field of epidemiology. The epigenome act as an ‘integrator’ of multiple signals - environmental exposures, germ-line genetic variation, stochastic events and possibly inherited non-germ-line phenomena (Relton and Smith, 2012). Possible influences of the multiple factors and their interaction or reverse causation pose numerous challenges. The situation is even more complicated by the fact that epigenetic aberrations can arise as a consequence of exposure to intermediate phenotypes. The published empirical data on NM-induced epigenetic effects are insufficient to confidently distinguish exposure-specific epigenetic changes on health outcomes.

Population-based studies face several limitations. The first is the high probability that any effect of NM exposure is unlikely to be quantitatively large and much more information is needed in this area to define the biological impact of these modest shifts in epigenetic patterns. It is also difficult to extrapolate from studies that show damage with high doses of NMs in the laboratory to observations in clinical settings, where exposures may be much lower, difficult to measure in individual organs, and may be intermittent and indeterminate. Another problem is the use of easily accessible sources of DNA such as peripheral blood, that do not accurately reflect epigenetic perturbations in the disease-specific target of interest. Moreover, changes in blood cell composition in response to exposure to NP could be a confounding factor, and should thus be taken into account. Furthermore, the majority of studies have separately evaluated only one type of the epigenetic marks. However, epigenetic processes closely interact, generating a self-reinforcing network of epigenetic events allowing sophisticated control of gene expression (Fuks, 2005). Alterations in multiple layers of epigenetic control mechanisms bring a challenge to establish what is the “normal” pattern within the dynamic variation in the epigenome. Also, the degree of epigenetic changes induced by NMs is strongly dependent on individual susceptibility. All these and other limitations currently hamper incorporation of an epigenetic component into NM safety assessment. New powerful cost-effective epigenomic technologies will allow us to

generate comprehensive maps of exposure-induced modifications on different levels of the epigenome and transcriptome.

Although epigenetic mechanisms have properties that make them ideal molecular intermediates of environmental effects, functional associations between NM exposure and disease outcomes are to date inconclusive. Recent technological advances are now facilitating the elucidation of epigenetic alterations, although there is still a need for maps of the human methylome and histone modifications in healthy and diseased tissues. Epidemiology and statistical approaches, including well-designed human studies and advanced statistical methods are urgently needed. Among the battery of epidemiological tools there is a requirement for statistical modelling that allows elucidation of the impact of inter-individual variation and changes over time, strengthens causal relationships and allows identification of particularly pertinent associations. However, they will generate large amounts of data that will require development of new bioinformatics tools. Particular attention should be paid to interpretation of these data and definition of adverse effects that pose a health risk, in contrast to adaptive change. Future epigenomic research may provide information for developing preventive strategies, including exposure reduction, as well as pharmacological, dietary or lifestyle interventions.

Conflict of interest

The authors declare that there are no conflicts of interest.

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