From INSTITUTE OF ENVIRONMENTAL MEDICINE
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CELLULAR EFFECTS OF NICKEL AND NICKEL OXIDE NANOPARTICLES: FOCUS ON MECHANISMS RELATED TO CARCINOGENICITY

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Stockholm 2018

Published by Karolinska Institutet.
Printed by E-print AB 2018

ISBN 978-91-7831-194-1

Cellular Effects of Nickel and Nickel Oxide Nanoparticles: Focus on Mechanisms Related to Carcinogenicity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to my mother

ABSTRACT

There has been a rapid increase in the production and useage of nanomaterials during the last decade and therefore it is of great importance to properly investigate safety of these materials. This thesis specifically focuses on nickel (Ni) and nickel oxide (NiO) nanoparticles (NPs). Ni metal particles have been classified by the International Agency for Research on Cancer as possibly carcinogenic to humans, Group 2B, while Ni compounds are classified in Group 1 i.e. carcinogenic to humans. There has been no specific classification for Ni containing nanoparticles yet and the risks or mechanisms of carcinogenicity are not fully elucidated. Human exposure to Ni containing nanoparticles can occur in occupational settings such as through nickel containing dust or through manufacturing of Ni NPs. The overall aim of this thesis was to increase the knowledge about the mechanisms underlying the carcinogenicity of Ni and Ni compounds as well as to particularly elucidate if Ni in the form of NPs (Ni and NiO) act via different mechanisms compared to those of soluble nickel ($NiCl_2$). In study I different models were employed to investigate genotoxicity and underlying mechanisms. Stronger genotoxic effects were observed for Ni and NiO NPs compared to $NiCl_2$ and oxidative stress was identified as an important mechanism for genotoxicity rather than direct DNA binding. In general, mutagenic effects were low however a significant increase was observed at one concentration of NiO. In study II genotoxicity and the involvement of calcium as a possible underlying mechanism was investigated. Chromosomal damage was induced by Ni and NiO NPs as well as $NiCl_2$ but cellular uptake was only observed for Ni and NiO NPs. A mechanism dependent on calcium and iron was identified for cyto- and genotoxicity. In study III inflammation and secondary genotoxicity was investigated by using macrophages and lung cells in a co-culture model as well as use of a conditioned media approach. Release of inflammatory cytokines from macrophages, exposed to Ni and NiO NPs, was found and evidence of secondary genotoxicity was observed. However it is still unclear what factors are responsible for these observations. In study IV the ability of Ni and NiO NPs as well as $NiCl_2$ to induce markers related to epithelial to mesenchymal transition (EMT) and a stem cell like phenotype was studied. Induction of both EMT and stem cell markers as well as cellular invasion/migration was found. Little to no differences was observed between the Ni and NiO NPs and soluble Ni. In conclusion both primary and secondary genotoxicity was observed following exposure to Ni and NiO NPs as well as mechanisms related to EMT and a stem cell like phenotype. NiO NPs were most potent in generating intracellular ROS and inducing DNA strand breaks. Ni and NiO NPs was shown to be taken up by the cells while ionic Ni was not (or limited) which lead to the hypothesis that Ni might act via mechanisms related to extracellular factors or interaction with cell membranes/receptors. The studies in this thesis have contributed to the knowledge in the field of different mechanisms related to carcinogenicity of Ni and NiO NPs.

SVENSK SAMMANFATTNING

Nickel är klassat som cancerframkallande men de underliggande mekanismerna är fortfarande inte helt klarlagda. I de flesta studier som hittills utförts har partiklar av nickel (Ni) eller nickel oxid (NiO) i mikrometerstorlek eller lösligt nickel studerats. Syftet med den här avhandlingen var att studera mindre partiklar, nanopartiklar (NPs), av Ni och NiO med fokus på en rad olika mekanismer som kan vara viktiga för utveckling av cancer. Resultaten jämfördes med effekten av lösligt nickel ($NiCl_2$).

Syftet med studie I var att undersöka genotoxicitet och mutagenicitet av Ni och NiO NPs samt att jämföra effekten med lösligt Ni i form av $NiCl_2$. Tre olika modellsystem användes för att undersöka detta: 1) exponering av humana bronkepitelceller (HBEC) samt analys av DNA strängbrott (comet assay och γ-H2AX-infärgning), 2) Exponering av sex olika embryonala mus-stamceller (mES), så kallade ”reporter cell lines” (ToxTracker) som fluorescerar då olika signalvägar av relevans för (geno)toxicitet och cancer aktiveras och 3) exponering av mES-celler följt av mutagenicitets-testning (Hprt-analys). Resultaten visade på ökning av DNA-strängbrott (comet assay) för NiO NP och vid högre doser även för Ni NP, medan inga effekter observerades för Ni joner/komplex från $NiCl_2$. Experiment med ToxTracker (reporter cell lines) visade på oxidativ stress som den huvudsakliga toxiska mekanismen samt en förändrad konformation hos proteiner (”protein unfolding”) vid cytotoxiska doser för alla tre Ni-exponeringarna. Oxidativ stress påvisades även i HBEC-cellerna efter NP-exponering. Det blev ingen induktion av rapportörcellerna som indikerar direkt DNA-skada eller påverkan på replikationsgaffeln (”stalled replikation forks”) av någon utav alla exponeringar (NiO NP, Ni NP och $NiCl_2$). En liten men statistiskt signifikant ökning av Hprt-mutationer observerades för NiO, men endast i en enda dos. Slutsatsen är att Ni och NiO NP visar mer uttalade (geno) toxiska effekter jämfört med Ni-joner/ komplex.

Syftet med studie II var att undersöka genotoxicitet av väl-karakteriserade Ni och NiO NPs i humana bronk-epitelceller (BEAS-2B) och att finna möjliga mekanismer. $NiCl_2$ användes för att jämföra effekterna av Ni-joner med den från NPs. BEAS-2B-celler exponerades för Ni och NiO NP samt $NiCl_2$ och upptag och dos i celler undersöktes med transmissionselektronmikroskop (TEM) och ICP-MS (”inductively coupled plasma mass spectrometry”). Nanopartiklarna karaktäriserades med avseende på ytkomposition, agglomerering och nickelfrisättning i cellmedium (ICP-MS). Celldöd (nekros/ apoptos) undersöktes genom annexin V/PI-infärgning (flödescytometri) och genotoxicitet testades genom analys av mikrokärnor, kromosomabberrationer och DNA stängbrott. Reaktiva syremolykyler (”ROS”) och kalcium mättes med fluorescerande prober. Resultaten visade att NPs effektivt togs upp av BEAS-2B-cellerna. Däremot observerades inget eller mindre upptag för Ni joner från $NiCl_2$. Trots skillnader i upptagning orsakade alla exponeringar (NiO, Ni NP och $NiCl_2$) kromosomskador. NiO NPs var mest potent i att orsaka DNA-strängbrott och generera intracellulär ROS. En ökning av intracellulärt kalcium observerades och manipulering av intracellulärt kalcium med hjälp av inhibitorer och kelatorer minskade kromosomskadorna. Kelering av järn skyddade också mot inducerad skada, speciellt för NiO och $NiCl_2$. Slutsatsen är att Ni och NiO NPs samt Ni-joner kan inducera kromosomskador och att mekanismen för cyto- och genotoxicitet kan vara kalciumberoende.

Syftet med stidie III var att undersöka inflammation och genotoxicitet av Ni och NiO NPs samt att undersöka möjligheten att testa sekundär (inflammationsdriven) genotoxicitet in vitro. Som en kontrollpartikel att jämföra med användes kristallin silika (SiO_2) eftersom den partikeltypen har visat på cancerframkallande egenskaper som föreslås vara inflammatoriskt drivna. Resultaten påvisade frisättning av inflammatoriska mediatorer, så som VEGF, MIF och Flt-3-ligand, då makrofager

(differentierade THP-1 celler) utsattes för partiklarna. Två olika system testades för att undersöka inflammationsdriven genotoxicitet. 1) epitelceller (HBEC) utsattes för medium från exponerade THP-1 celler (”Conditioned media”, CM) och DNA-strängbrott hos HBEC analyserades med kometmetoden. 2. Makrofager utsattes för partiklarna då de samodlades tillsammans med HBEC celler (enbart makrofagerna var direkt utsatta för partiklarna) och kometmetoden användes därefter för att undersöka DNA-strängbrott hos HBEC. I båda fall visade resultaten visade på en ökning av DNA strängbrott. Slutsatsen är att Ni och NiO kan orsaka sekundär genotoxicitet och denna effekt kan studieras med dessa två testsystem.

Syftet med studie IV var att undersöka molekylära mekanismer som är relevanta för cancer med fokus på ”epitelial to mesenchymal transition” (EMT) och förvärv av en stamcellslik fenotyp. Effekter orsakade av Ni i form av NPs (Ni och NiO) jämfördes med Ni-joner ($NiCl_2$). BEAS-2B exponerades för NiO NP, Ni NP och $NiCl_2$ i 48 h och förändringar i genuttryck testades (qPCR) liksom förändringar i proteinnivåer (In-cell western, flödescytometri och flourescensmikroskopi) av markörer relevanta för EMT, stamcellslik fenotyp samt tumörsupresseorgener. Funktionella analyser utfördes också för att se om cellerna fick en större benägenhet att migrera (”scratch wound healing assay” och ”invasion/migration assay”). Resultaten visade att alla Ni-exponeringar ledde till minskat uttryck av tumörsuppressorgener, förändringar i markörer förknippade med EMT inklusive minskat uttryck av E-cadherin, ökad förmåga att migrera samt förändringar kopplade till en stamcellsfenotyp, såsom minskat uttryck av celladhesionsmolekylen CD24. Vi drar slutsatsen att Ni och NiO NP orsakar förändringar kopplade till EMT men att effekterna inte var nano-specifika effekter då exponering för Ni-joner visar likande effekter.

LIST OF SCIENTIFIC PAPERS

I. **Åkerlund E**, Cappellini F, Di Bucchianico S, Islam S, Skoglund S, Derr R, Odnevall Wallinder I, Hendriks G, Karlsson HL. Genotoxic and mutagenic properties of Ni and NiO nanoparticles investigated by comet assay, γ-H2AX staining, Hprt mutation assay and ToxTracker reporter cell lines. Environmental and Molecular Mutagenesis. 2018. 59(3):211-222

II. Di Bucchianico S, Gliga AR, **Åkerlund E**, Skoglund S, Odnevall Wallinder I, Fadeel B, Karlsson HL. Calcium-dependent cyto- and genotoxicity of nickel metal and nickel oxide nanoparticles in human lung cells. Particle and Fibre Toxicology. 2018. 15(1):32

III. **Åkerlund E**, Islam S, Alfaro-Moreno E and Karlsson HL. Inflammation and (secondary) genotoxicity of Ni and NiO nanoparticles. [Manuscript]

IV. **Åkerlund E**, Di Bucchianico S and Karlsson HL. Ni and NiO nanoparticles cause changes linked to epithelial-mesenchymal transition (EMT) a stem cell like phenotype in epithelial lung cells. [Manuscript]

CONTENTS

LIST OF ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
BEAS	Human bronchial epithelial cells
Blvrb	Seipin lipid droplet biogenesis associated
Btg2	B-cell translocation gene 2
Bscl2	Biliverdin reductase B
CSC	Cancer stem cells
CD	Cluster of differentiation
CFE	Colony forming efficiency
Ddit3	DNA damage-inducible transcript 3
DDR	DNA damage response
DNA	Deoxyribonucleic acid
EGCG	Epigallocatechin-3-gallate
EGF	Epidermal growth factor
ELISA	Enzyme linked immuosorbent assay
EMT	Epithelial to Mesenchymal Transition
EPC	Endothelial progenitor cell
FPG	Formamidopyrimidine DNA glycosylase
FSC	Forward scatter
GFP	Green fluorescent protein
GSH	Glutathione
H_2DCF-DA	Dihydrodichlorofluorescein diacetate
HBEC	Human Bronchial Epithelial cells
HIF1α	Hypoxia-inducible factor 1α
HPF	Hydroxyphenyl fluorescein
H_2O_2	Hydrogen peroxide

HPRT	Hypoxanthine phosphorybosyl transferase
IARC	International Agency for Research on Cancer
ICP-MS	Inductively coupled plasma mass spectrometry
IFN-γ	Interferon gamma
IL	Interleukin
ISDD	*In vitro* Sedimentation, Diffusion and Dosimetry
MAPK	Mitogen activated protein kinase
mES cells	mouse embryonic stem cells
MET	Mesenchymal to epithelial transition
MIF	Migration inhibitory factor
mRNA	Messenger RNA
NaCl	Sodium chloride
NF-κB	Nuclear factor-κB
Ni	Nickel
$NiCl_2$	Nickel chloride
NiO	Nickel oxide
NiS	Nickel sulfide
Ni_3S_2	Nickel subsulfide
$NiSO_4$	Nickel sulfate
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NP	Nanoparticle
NSCC	Non-small cell carcinoma
NSCLC	Non-small cell lung carcinoma
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
O_2	Dioxygen

$O_2^{\cdot-}$	Superoxide radical
PARP	Poly ADP-ribose polymerase
PCCS	Photon cross-correlation spectroscopy
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rtkn	Rhotekin
SCLC	Small cell lung carcinoma
SIRT1	Sitruin 1
TEM	Transmission electron microscopy
TNFα	Tumour necrosis factor alpha
Srxn1	Sulfiredoxin 1
SSC	Side scatter
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
TiO_2	Titanium dioxide
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
XPS	X-ray photoelectron spectroscopy
ZEB1	Zinc finger E-box-binding homeobox 1
8-OHdG	8-oxo-7,8-dihydro-2' -deoxyguanosine

1 INTRODUCTION

The overall aim of this PhD project is to increase the knowledge about the mechanisms underlying the carcinogenicity of nickel compounds and particularly to elucidate if nickel (Ni) in nanoparticle-form (nickel NP and nickel oxide NP) act via different mechanisms compared to soluble nickel ($NiCl_2$). Thus, this thesis includes an introduction to the field of cancer, particularly lung cancer, and how various environmental factors increase the risk for lung cancer. Furthermore, since most research performed to date is about micron-sized Ni compounds as well as soluble Ni, the knowledge about human exposure, carcinogenicity and underlying mechanisms of these compounds will be discussed.

1.1 CANCER

Cancer is one of the leading causes of mortality and morbidity in the world. In 2018 the cancer burden has risen to 9.6 million cancer related deaths and 18.1 million new cases. Lung, breast and colorectum are the top three cancer forms when it comes to incidence and are ranked among the top five in terms of mortality. Because of its poor prognosis, lung cancer accounts for the largest number of deaths, corresponding to 1.8 million which is 18.4% of the total deaths. The most commonly diagnosed (men 14.5%, women 8.4%) and leading cause of cancer death in men (22%), is lung cancer (IARC 2018). New cancer cases are expected to rise by 70% in the next couple of decades. There are five leading lifestyle factors that causes about a third of the cancer cases; high body mass index, lack of physical activity, low fruit and vegetable intake, tobacco use and alcohol use. The most important factor is tobacco use which causes around 70% of global lung cancer deaths and 20% of global cancer related deaths. Up to 20% of cancer related deaths in low- and middle-income countries are because of viral infections such as human papilloma virus, hepatitis B and C. More than 60% of the world's total new annual cases occur in Africa, Asia and Central and South America, which account for 70% of the world's deaths related to cancer (WHO 2016).

Cancer, malignant tumours or neoplasms, are terms used to describe a large group of diseases which can affect any part of the body. Cancer is in short an uncontrolled fast proliferation of abnormal cells which can metastasize and spread thought the body to different organs. In most cases the cause of death is not the primary tumour but the metastasis (WHO 2016). The six hallmarks of cancer proposed in 2000 were; sustaining proliferative signaling, activating invasion and metastasis, evading growth suppressors, enabling replicative immortality, inducing angiogenesis and resting cell death (Hanahan and Weinberg 2000). In later years researchers has come to identify two emerging hallmarks of cancer: avoiding immune destruction and deregulating cellular energetics as well as two enabling characteristics: tumour promoting inflammation and genome instability and mutation (Hanahan and Weinberg 2011). The hallmarks of cancer are summarized in figure 1.

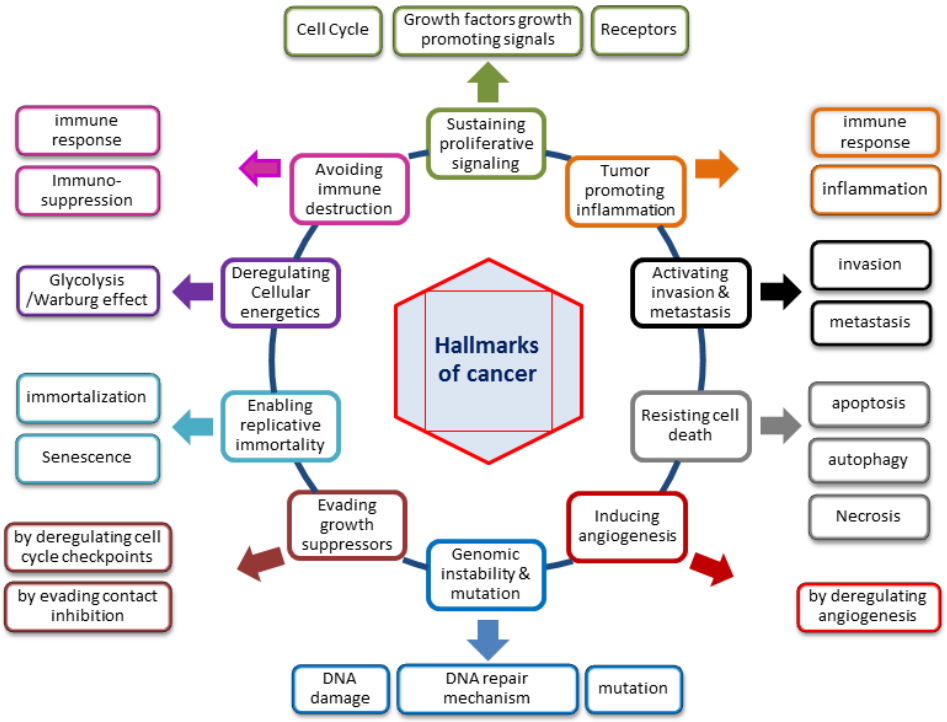

Figure 1. Hallmarks of cancer (Hanahan and Weinberg 2011).

1.1.1 Lung Cancer

Lung cancer is divided into two groups; non-small cell carcinoma (NSCC) and small cell carcinoma (small cell lung carcinoma, SCLC). NSCC accounts for 80% of the cases and SCLC for 20%. SCLC is highly aggressive and is most commonly treated non-surgically while NSCC can be managed with a combination of adjuvant therapy and surgery. SCLC is caused by smoking in virtually all the cases. NSCC are divided into subgroups which include adenocarcinoma 60%, squamous cell carcinoma 20% and large cell carcinoma (Zheng 2016).

Despite new treatments for the disease the global situation is worse than before. Most lung cancers are discovered at a late stage and most patients with lung cancer eventually die from it. A big risk factor for lung cancer is the world wide spread addiction to smoking and accounts for 80% of the cases in men and 50% in women. The risk of lung cancer increases with dose and duration where duration increase the risk more than number of cigarettes smoked per day (Mao et al. 2016).

1.1.2 DNA damage

DNA is damaged constantly by insults of various environmental factors e.g. ionizing radiation or by intracellular factors such as reactive oxygen species (ROS). A signaling network that manages DNA damage response including cell cycle checkpoints and DNA

repair pathways protects the genome in normal cells. However, cancer are thought to rise up from accumulation of genetic alterations that results in survival advantages and growth (Broustas and Lieberman 2014). DNA damage can occur in the following forms; single strand breaks, double strand breaks, base modifications, base mismatch, DNA-protein crosslink, intrastrand crosslink and interstrand crosslink (leading to stalled replication forks). The DNA damage response (DDR) can recognize and process, with help of different proteins, the different types of DNA damage (Hosoya and Miyagawa 2014). Genomic instability is considered as a hallmark of cancer (Hanahan and Weinberg 2011). Downregulation of DDR pathways such as the ones that control p53, ataxia telangiectasia and Rad3-related, kinases ataxia telangiectasia mutated and ataxia telangiectasia can give rise to genomic instability. There are also six DNA repair pathways; nucleotide excision repair, base excision repair, homologous recombination repair, DNA mismatch repair, translesion DNA synthesis as well as non-homologous end joining. Defects in any of them can also lead to genomic instability (Hosoya and Miyagawa 2014).

1.1.3 Inflammation and Cancer

The link between cancer and inflammation was first suggested by Rudolf Virchow in 1863. He noted leucocytes in neoplastic tissues and stated the hypothesis that this reflected the origin of cancer at chronic inflammation sites (Balkwill and Mantovani 2001). Since then, the field has progressed and tumour promoting inflammation was included as a cancer hallmark in Hanahan and Weinberg (2011) when the list was updated.

The link between cancer and inflammation can be described by two different pathways: the intrinsic and extrinsic pathway. The intrinsic pathway includes genetic alterations as for instance activation of oncogenes by mutation, inactivation of tumour suppressor genes, chromosomal rearrangement or amplification. Cells that are transformed can create an inflammatory tumour microenvironment, by producing inflammatory factors, where there was no existing inflammation initially. In the extrinsic pathway, already existing inflammation or infection in certain parts of the body, e.g. prostate, colon and pancreas, enhance the risk of cancer development. These two pathways can meet and result in transcription activation in tumour cells of factors such as signal transducer and activator of transcription 3 (STAT3), nuclear factor-κB (NF-κB) and hypoxia-inducible factor 1α (HIF1α). The mentioned transcription factors are in charge of production of inflammatory factors such as chemokines and cytokines as well as a cyclooxygenase 2 which generates production of prostaglandins. This, in turn, leads to the recruitment of leukocytes where the same key transcription factors become activated by cytokines, resulting in production of more inflammatory mediators and generation of a cancer inflammatory microenvironment (Mantovani et al. 2008).

Inflammation can in itself generate oxidative stress which in turn can cause more inflammation (Fernandes et al. 2015). ROS and reactive nitrogen species (RNS) can be produced by inflammatory cells which can lead to induction of DNA damage, enhancement of mutation rate as well as an increase of genomic instability (Waris and Ahsan 2006).

Macrophages can release ROS in a process called respiratory burst in response to both microbes (Gwinn and Vallyathan 2006) and particle encounter (Beck-Speier et al. 2005).

1.1.4 Epithelial to Mesenchymal Transition and Cancer

Epithelial to Mesenchymal Transition (EMT) is a process where cells with epithelial phenotypic properties transform into cells with mesenchymal phenotypic properties including loss of cell polarity, loss of cell-cell adhesion and the acquisition of migratory and invasive traits. Cells can also undergo mesenchymal to epithelial transition (MET) which is the reverse process of EMT (Thiery et al. 2009). EMT occurs in embryonic development, wound healing, as well as cancer and are divided into tree subtypes. Type I takes place in gastrulation in neural crest cells, produced by the mesoderm and ectoderm, which undergoes MET to become epithelial cells in organs. Type II can occur in wound healing and lead to fibrosis during persistent inflammation. Type III occurs in cancer where the cancer cells can use part of the EMT type II program to migrate and invade distant sites in the body, thereby cause metastasis. At the distant site the cancer cells are believed to undergo MET and then start to proliferate to grow the metastasis (Scanlon et al. 2013).

Loss of E-cadherin, which is a key component of adherens junctions, is a critical event that causes dissolution of the cell-cell contacts during EMT. Lysosomal degradation of E-cadherin and endocytosis can be the initial stages of EMT. Metastatic progression and EMT are often associated with reversible E-cadherin downregulation involving either repression by EMT-inducing transcription factors or hypermethylation of the *CDH1 (the E-cadherin gene)* promoter (May et al. 2011). EMT is also characterized by a decrease in expression of other epithelial markers such as Claudins and Occludin as well as an increased expression of mesenchymal markers, such as Fibronectin, Vimentin, N-cadherin and also transcription factors such as SNAIL, SLUG and Twist (Aroeira et al. 2007), shown in figure 2.

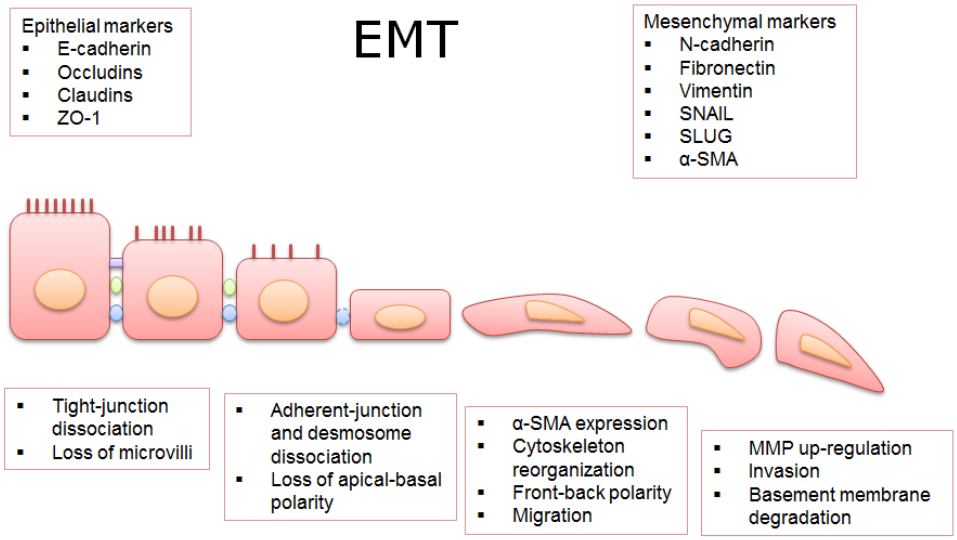

Figure 2. Overview on EMT recreated from (Aroeira et al. 2007).

1.1.4.1 EMT and CSC

Another topic discussed in conjunction with EMT and cancer is stem cell markers. The cancer stem cell theory states that tumour progression is driven by a small subpopulation of cancer cells namely cancer stem cells (CSCs) even called tumor-initiating cells. These CSCs is defined by two characteristics: the ability to self-renew and the ability to regenerate the phenotypic heterogeneity of the parental tumor (May et al. 2011). CSCs have been involved in initiation and sustentation of growth of primary tumours as well as of metastases (Abraham et al. 2005; Al-Hajj et al. 2003; Ginestier et al. 2007; Liu et al. 2007; Sheridan et al. 2006). CSCs are also responsible for cancer unlimited growth, generation of different populations of cancer cells and chemotherapy resistance (Floor et al. 2011).

It was found that EMT induction in immortalized human mammary epithelial cells leads to de novo expression of stem cell markers and gain of functional stem cell properties such as mammosphere formation. EMT in non-tumorigenic, immortalized human mammary epithelial cells (HMLEs) was generated and it was found that most mesenchymal like cells acquired a $CD44^{high}/CD24^{low}$ expression pattern. Neoplastic mammary stem cells have been associated to this phenotype. This finding suggests a link between EMT and cancer stem cells (Mani et al. 2008). Another study recently found that $CD44^{high}/CD24^{low}$ cells isolated from the oral cancer cell lines express genes related to stem cells, show characteristics of EMT and this population could give rise to all other cell types. Typical cancer stem cell phenotypes were also confirmed in $CD44^{high}/CD24^{low}$ cells such as migration and invasion, increased colony formation and sphere forming ability (Ghuwalewala et al. 2016).

1.1.5 Environmental Pollution and Lung Cancer

One environmental risk factor for lung cancer is air pollution which is found in both indoor and outdoor air. Examples of sources of outdoor air pollution are cars, industrial waste burning and heating systems. Polycyclic aromatic hydrocarbons as well as metals such as Ni, arsenic and chromium are carcinogens that are generated from combustion of fossil fuels. Indoor air pollution can originate from cooking fumes, and benzene and formaldehyde can be formed from home décor and building materials. Another source of indoor exposure can be radon which comes from soil and building materials. Radon is probably the second most common cause of lung cancer after smoking. Twelve occupational exposure factors have been identified by The International Agency for Research on Cancer (IARC) as being carcinogenic to human lung: Ni, aluminum production, arsenic, asbestos, bis-chloromethyl ether, beryllium, cadmium, hexavalent chromium, coke and coal gasification fumes, crystalline silica, radon, and soot. Other risk factors for lung cancer can be radiation as well as diet, where intake of more fruits and vegetables has been shown to decrease the risk (Malhotra et al. 2016; Mao et al. 2016). About 15-20% of all the lung cancer cases are caused by occupational carcinogens and ionization radiation (Didkowska et al. 2016).

1.1.6 Nickel and Lung Cancer

Nickel is a metallic compound existing in various mineral forms and is a naturally occurring element. It is the 24th most abundant element found in the crust of earth. It is mainly found in soil and sediment, and the physicochemical properties of the soil affects its mobilization (Cameron et al. 2011). Inorganic Ni compounds are the ones mainly regarded as toxicologically relevant. There are four classes of inorganic Ni species based on analytical procedure: soluble, sulfidic, metallic and oxidic Ni. This general classification is widely used in studies analyzing Ni particulate matter in air of occupational settings (Schaumloffel 2012).

Ni metal particles are classified by IARC as possibly carcinogenic, Group 2B, whereas Ni compounds e.g. high temperature green NiO, are classified as a human carcinogen via inhalation exposure, Group 1Ai (IARC 1990). A variety of mechanisms are suggested to be responsible for Ni induced cancer development, but the molecular mechanisms of Ni carcinogenicity are not yet fully elucidated (Schaumloffel 2012). The actual carcinogenic species is believed to be ionic Ni (Ni^{2+}) because it can bind to cellular components including DNA and nuclear proteins (Shen and Zhang 1994). The binding of Ni ions to DNA is considered to be weak but the binding to nuclear proteins, such as protamines and histones, is with high affinity (Kasprzak et al. 2003; Oller et al. 1997). It is also suggested that Ni ions can inhibit enzymes that are required for the repair of DNA as well as enhance the genotoxic effects of X-rays and ultraviolet light (Hartwig et al. 1994).

The National Toxicology Program (NTP) performed long time inhalation exposure studies in mice and rats during 2 years. The rats were exposed to 0, 0.62, 1.25, or 2.5 mg NiO/m^3 and mice were exposed to 0, 1.25, 2.5, or 5 mg NiO/m^3 by inhalation for 6 h/day, 5 days/week for 104 weeks. The outcome in male and female rats was: some evidence of carcinogenic activity of NiO. This was based on increased incidences of combined alveolar/bronchiolar adenoma or carcinoma and increased incidences of combined benign or malignant pheochromocytoma of the adrenal medulla. For the mice the results were: equivocal evidence of carcinogenic activity of NiO in the female mice and in the male mice there were no evidence. In the female mice this was based on marginally increased incidences of alveolar/bronchiolar adenoma in 2.5 mg/m^3 and of alveolar/bronchiolar adenoma or carcinoma (combined) in 1.25 mg/ m^3 (National Toxicology 1996).

1.1.6.1 Nickel and Mutagenicity

The mutagenic potential of Ni compounds is generally considered to be low, despite several reports of the chromatin- and DNA damage found in cells and tissues exposed to Ni. The results that lead to this conclusion come from mutagenesis assays performed in fruit fly, bacteria and mammalian cells (Biggart and Costa 1986; Fletcher et al. 1994; Kargacin et al. 1993; Lee et al. 1995; Rodriguez-Arnaiz and Ramos 1986). Nevertheless, there are data that suggest that Ni could be a potent co-mutagen with alkylating mutagens in some *S typhimurium and E. coli* tester strains (Dubins and LaVelle 1986).

Parental exposure to certain metals can increase the risk of cancer in the progeny, according to epidemiological studies. Exposure to Ni is suspected to cause pro-mutagenic damage to sperm DNA (Cameron et al. 2011). One study showed that Ni(II) could mechanistically be involved in reproductive toxicity and carcinogenicity of metals (Liang et al. 1999). Another study showed that exposure to Ni chloride in male mice resulted in a transient amount of Ni^{2+} in the testes as well as chromosomal aberrations and reduced sperm count (Cameron et al. 2011).

A previous study demonstrated that $NiCl_2$ and black NiO did not induce mutations in V79-cells but G12 (a transgenic cell line) showed a strong response to the insoluble Ni compounds (NiO, NiS and Ni_3S_2) in the *HPRT*-mutation assay (Kargacin et al. 1993). The G10 transgenic cell line also showed a strong response in regards to mutations after exposure to NiO, NiS and Ni_3S_2. $NiCl_2$ were also potent in inducing mutations but to a lesser extent (Klein et al. 1994).

1.1.6.2 Epigenetic mechanisms

Gene expression is not only determined by base sequence of DNA, but also depends on dynamic states of the chromatin. DNA methylation and histone posttranslational modifications are the two main groups of epigenetic mechanisms that affects gene expression at the chromatin level (Arita and Costa 2009). The best understood and most common epigenetic modification in DNA is methylation. The molecular process of DNA methylation is when a methyl group is added to the 5′ position of the cytosine ring on a CpG dinucleotide to create 5-methylcytosine (Miller et al. 1974). Methylation of the promoter region can be used to turn on and off gene transcription and methylation of a gene promoter in general leads to repression of transcription (Jones 1999).

Changes in DNA methylation leading to gene expression inactivation after Ni exposure was initially found in the Chinese hamster G12 cell line. This cell line possesses a copy of the bacterial *gpt* transgene near the telomere of chromosome 1 (Lee et al. 1995). An *in vivo* study showed changes in DNA methylation after exposure to Ni sulfide and tumours were induced. Hypermethylation of the promoter of gene p16, which is a tumour suppressor gene, was found in all tumours in the mice (Govindarajan et al. 2002). Another study showed a correlation between Ni urinary levels and DNA methylation in the promoter of the tumor suppressor gene p15 in Ni exposed workers (Yang et al. 2014).

1.1.6.3 Particles vs Ions: Ni bioavailable theory

The so-called “nickel ion theory” states that Ni of all forms could lead to an increased risk of lung cancer, with the following order lowest to highest risk: water-soluble Ni, metallic and sulfidic Ni, and last oxidic Ni. The nickel ion bioavailability model is refined from the nickel ion theory. This model states that: “the presence of nickel ions in a substance is not sufficient for that substance to be a complete carcinogen”. To act as a carcinogen, Ni ions that become bioavailable at the nucleus of epithelial lung cells must be released from the substance. It must also reach the cell in sufficient amounts and the cell has to survive in order for the

substance to cause carcinogenesis. The degree of bioavailability will decide the carcinogenic potency of the Ni ion releasing substance. Interaction of many different factors influences the Ni ion bioavailability at epithelial cell nuclear sites. Such factors can be; target cell uptake, clearance, intracellular dissolution and respiratory toxicity, which also differ between different forms of Ni. This could mean that there is a threshold for Ni initiated carcinogenesis. For some compounds a "practical" threshold for carcinogenesis is likely such as weakly genotoxic substances. For compounds like these, secondary mechanisms of carcinogenesis could be of importance. Workers exposed to sulfidic and oxidic Ni compounds have an increased incidence of lung cancer, but this does not apply for workers exposed to metallic Ni or water soluble Ni compounds alone, which is evidence that supports the nickel ion bioavailability model (Goodman et al. 2011).

1.1.6.4 Nickel and EMT

Only a few studies have been performed regarding Ni and EMT to this date. One study showed that $NiCl_2$ and $NiSO_4$ can induce expression of EMT markers and E-cadherin promoter hypermethylation through ROS generation. Both $NiCl_2$ and $NiSO_4$ were shown to reduce expression of E-cadherin in BEAS-2B cells. $NiCl_2$ were also shown to decrease E-cadherin in lung cancer cell lines. Longer exposure (6 or 9 days) to a lower dose of $NiCl_2$ was shown to decrease E-cadherin and increase fibronectin expression. Upregulation of Hif1α was observed after $NiCl_2$ exposure. Transcription factors SNAIL, SLUG and HiF1a was shown to bind to the E-cadherin promoter after exposure to $NiCl_2$. The suggested mechanism for EMT in this study was via ROS and exposure to $NiCl_2$ can lead to epigenetic changes in the E-cadherin promoter (Wu et al. 2012).

Another study showed that exposure to $NiCl_2$ induced a persistent mesenchymal phenotype in BEAS-2B cells through epigenetic activation of ZEB1. Transcriptional changes persisted even after the exposure was removed and depletion of ZEB1 resulted in attenuation of EMT in exposed cells. Both acute (72 h) and long term (6 weeks) exposure was performed. Decrease in E-cadherin and Claudin 1 as well as increase in fibronectin expression was observed after acute exposure. E-cadherin levels were also decreased after long term exposure in BEAS-2B as well as in the RT4 cancer cell line. Increased invasive ability was also observed in BEAS-2B and RT4 cells after long term exposure (Jose et al. 2018).

Another study showed that exposure to $NiCl_2$ (1 mM 48 h) down regulated *E-cadherin* and *Claudin7* and strongly upregulated *Snai2* but not *Snai1*. The same study also found that SFMBT1 (belongs to the malignant brain tumor (MBT) domain-containing protein family that is critical in chromatin regulation) is essential for the induction of EMT by $NiCl_2$ (Tang et al. 2013).

1.1.6.5 Nickel Exposure in Humans

Exposure of metals to humans is common due to the wide usage in industries and also the persistence of metals in the environment. Ni industries, trash incinerators, oil and coal burning power plants have been contributing to release into the environment. Ni existing in

the atmosphere is removed from air by rain or snow alternatively by attaching to small dust particles which further settle to the ground. Ni that sediment or is released from industrial wastewater into the soil can bind to manganese or iron particles. Ni is primarily used in stainless steel production as an alloy and this usage accounts for 80% of the Ni use. It can also be used in battery production and plating which accounts for 10% of the usage. Around 5% of the primary Ni is used in printing inks and applications of foundary. Different factors such as dose, route of exposure, and solubility of the Ni compound influences the toxicity. The major route for exposure to toxicity induced by Ni is lung inhalation. Ni can also be absorbed through the skin or ingested, and the primary target organs are the lungs and kidneys. Other organs that can be affected, but to a lesser extent, are; spleen, liver, heart and testes (Cameron et al. 2011).

Humans are exposed to Ni via ambient air, food consumption, drinking water or by smoking tobacco. Intake via food and drinking water stands for the highest intake of Ni and Ni compounds in the general population (Schaumloffel 2012). Other sources of exposure can be via skin absorption caused by direct skin contact from jewelry and coins. Another exposure could be from artificial body parts such as endoprostheses, bone-fixing plates and screws, where Ni containing alloys are used (Kasprzak et al. 2003; Schaumloffel 2012).

Non-smokers inhale about 0.1-1 μg Ni per day. Smoking of cigarettes increases exposure to Ni by 0.4 microgram per day. Inhalation of Ni is the primary source of exposure for workers in the Ni industries. Occupationally exposed individuals have higher levels of Ni than the general population. The Ni amount which is likely inhaled by the general population ranges from 0.1-0.25 mg Ni per day, while 0.3 to 0.8 mg Ni per day in Ni refining operations are likely to be inhaled. Depending on the industry, the amount of inhalation ranges from < 0.02 to 1.0 mg Ni per day (Cameron et al. 2011). About 2% of the workers in industries related to Ni are exposed to concentrations of airborne Ni containing particles in the range of 0.1 to 1 mg/m^3 (Kasprzak et al. 2003). For powder metallurgy operations the average airborne Ni concentrations have been reported to exceed 1.0 mg/m^3 (Cameron et al. 2011).

1.2 NANOPARTICLES

Nanomaterials can be described as materials “with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale”, where the nanoscale most often is considered to be between 1-100 nm. Nanomaterials are used for a vast number of purposes in the industry today, for instance in tires, stain-resistant clothing, sporting goods, cosmetics, sunscreens, and electronics. The usage is also increasing in medical applications like imaging, diagnosis, and drug delivery (Nel et al. 2006). The number of products containing nanomaterials is to this day approximately 3000 according to the Danish database (nanodb.dk 2018).

Nanoparticles (NPs) are often characterized as a material that has one or more dimensions between 1-100 nm. However this definition has been debated but in 2011 EU recommended the following definition of a nanomaterial: *“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" (2011/696/EU 2011).

NPs hold specific properties, compared to their non nano sized equivalent, and can induce nano specific toxic effects. They harbor unusual properties due to their size, chemical composition and shape. The small size of NPs can be similar to biological molecules, for instance viruses or proteins, which allows them to enter tissues, cells and organelles. In nanotoxicology the dose is of course important, but the size is also crucial because it affects the surface to mass ratio, which in turn has an impact on surface reactivity and chemistry (Fadeel and Garcia-Bennett 2010). NPs can travel through the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria and trigger injurious responses (Nel et al. 2006).

Exposure to NPs can occur through inhalation, ingestion or skin contact. Due to their small size, penetration and deposition of NPs can occur deeper in the alveolar region in the lungs as compared to larger particles. Information about NP lung deposition is important in order to estimate the dose received by the organism after inhalation. Diffusion is the main mechanism for deposition after inhalation of NPs and occurs due to collision of the NPs with air molecules. Mechanisms that apply for larger particles do not apply for NPs except for electrostatic precipitation which only occurs when NPs harbor electric charge (Oberdorster et al. 2005). Based on a model by the International Commission on Radiological Protection (ICRP 1994) particles of different size deposits differently in the nasopharyngeal, tracheobronchial and alveolar region of the respiratory tract. For instance, the model predicts that 90% of 1 nm NPs deposits in the nasopharyngeal part, 10% in the tracheobronchial part while almost none in the alveolar part. The prediction for 5 nm NP deposition was more or less an equal distribution in all three parts. The 20 nm NPs was predicted to deposit around 50% in the alveolar part and 15% in the other two parts, respectively (figure 3). After deposition, the NPs can translocate to extrapulmonary sites and end up in other organs via different routes such as: transcytosis through epithelial cells into the interstitium, and hence get access to the blood stream directly, or via the lymphatic system. Another, not generally recognized, route is via sensory nerve endings which are embedded in airway epithelia and thereafter axonal translocation to CNS and ganglionic structures (Oberdorster et al. 2005). It has been observed that particles can take a paracellular route between the cells by disrupting the tight junctions themselves or if some other agent cause them to disrupt and thereafter enter the blood stream (Puisney et al. 2018). It has also been shown that particles can translocate from the nose via the olfactory bulb to the brain (Oberdorster et al. 2004).

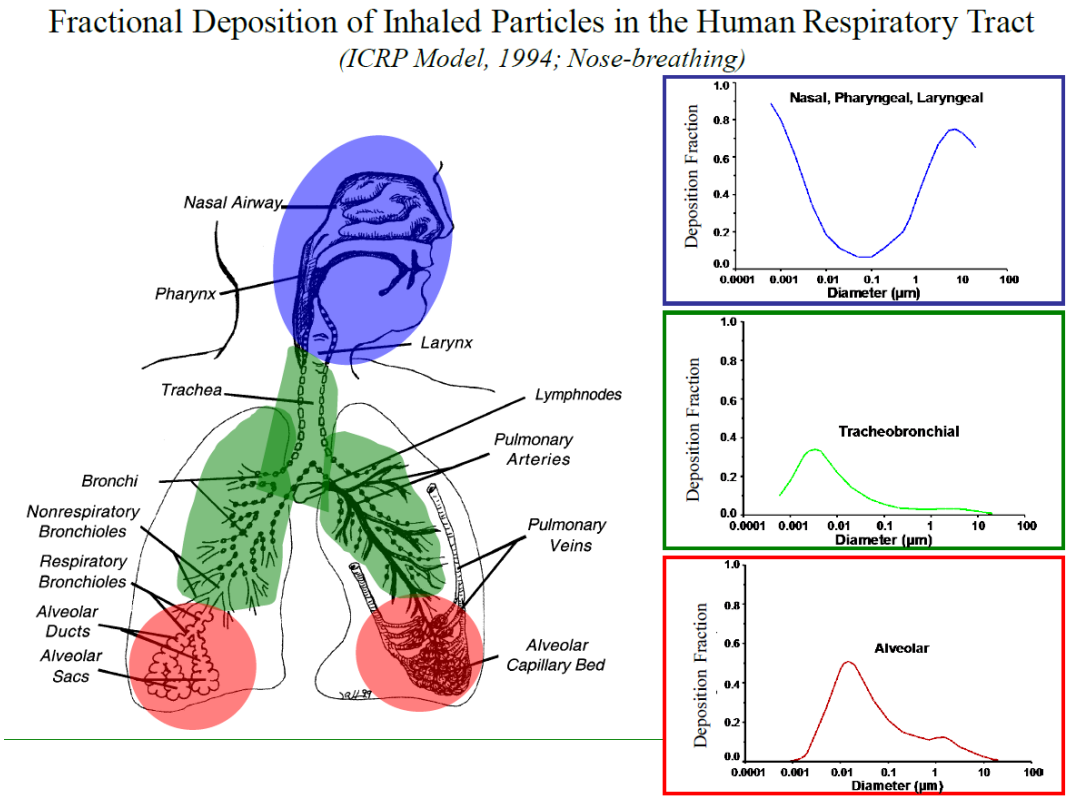

Figure 3. Fractional deposition of inhaled particles in the human respiratory tract. From (Oberdorster et al. 2005) with corrected modifications. (Permission received from Günter Oberdörster.)

Clearance of particles in the respiratory tract can occur either via chemical clearance or physical translocation of particles by different processes. The physical clearance processes are different for each of the tree regions of the respiratory tract and include; mucociliary movement, macrophage phagocytosis, epithelial endocytosis, interstitial translocation, lymphatic drainage, blood circulation and sensory neurons. The chemical clearance processes are: dissolution, leaching and protein binding, and they can occur in any of the three region (Oberdorster et al. 2005).

An important aspect of NPs is the so called “transport principle” (Krug and Wick 2011) or the “Trojan horse effect”. Metal ions are normally transported across the cell membrane and this process is well-regulated, but when the metal comes in contact with the cell in NP form this regulation is circumvented via several endocytotic mechanisms. When the particles have entered the cell, they may undergo dissolution within acidic cell compartments, this occurs in regard to many metal and metal oxide NPs. In other words, the particle structure in the form of a NP acts as a Trojan horse which sneaks into the cells and once inside releases ions, which results in toxic events (Cronholm et al. 2013; Stark 2011).

An important property that particles harbor is the inverse relationship between size of the particle and the number of expressed molecules on the surface. When the size of a particle decreases, the surface area further increases, hence displaying more surface atoms which in turn makes them more reactive. Engineering of nanomaterials can change physicochemical and structural properties; for instance, a decrease in size can lead to a number of material interactions which in turn leads to toxicological effects. The impact of these changes depends

on the composition of the material, for instance surface groups can change the nanomaterial from hydrophobic to hydrophilic or lipophilic to lipophobic. Interaction of electron acceptor or donor active sites (physically or chemically activated) with molecular dioxygen (O_2) is an example of surface properties that can lead to toxic events. If an electron is stolen from the dioxygen molecule the formation of free radicals will occur, namely the superoxide radical ($O_2^{\cdot-}$), which will generate ROS. This can occur in both single component material and also in the presence of transition metals on the surface of engineered nanomaterials e.g., Fe and vanadium. The best developed paradigm for toxicity of NPs is ROS generation. Other properties could also affect physicochemical and transport properties and possibly amplify size effects such as shape, surface coating, aggregation, and solubility (Nel et al. 2006).

1.2.1 Studying Toxicity of Nanoparticles

An important aspect of nano toxicology is proper characterization of the nanomaterial. This is important to be able to correlate the particle characteristics with the measured toxicological or biological outcome as well as setting a reference point to be able to compare the results with other studies. The properties that dictate the uniqueness of the material are chemical composition, size and shape as well as possible coating. There are three phases of particle characterization; primary in their dry state, secondary when particles are in solution or suspended in liquid and tertiary which is performed when the particles are in interaction with cells *in vitro* or *in vivo*. Preferably characterizations needs to be performed using more than one technique (Sayes and Warheit 2009).

Another important aspect is the dosimetry, meaning the accurate measure of the dose or the amount of, in this case, particles that comes in contact with the target. Different dose metrics have been used within nano toxicology including: µg/ml, cm^2/ml, µg/cm^2 and particle number/ml. There have been further statements that the nominal dose might not be the accurate dose that is delivered to the cells, or the intracellular dose (Lison et al. 2014). To estimate the delivered dose mathematical models can be used as a tool. For instance the ISDD (*In vitro* Sedimentation, Diffusion and Dosimetry) model can be used to make predictions about the power of *in vitro* systems and improve accuracy which will make it easier to design studies that better relate to human exposure (Hinderliter et al. 2010). However, the intracellular dose is not always relevant as some types of NPs might act extracellularly by for instance release of ions. Uptake should also be considered as different cells in different states might take up particles differently (Lison et al. 2014). Another problem is that some studies use exaggerated doses that are too high to be of relevance in a realistic exposure scenario in humans. This could be the result of the desire to publish positive results. Hence it is important to consider relevant doses both *in vivo* and *in vitro* (Krug and Wick 2011).

Another issue is that some NPs can harbor some intrinsic properties which can interfere with classical toxicological assays and possibly interfere with the results. Some examples are; interferance with MTS cell viability assay (Doak et al. 2009), quenching of florescent dyes (Sabatini et al. 2007), quenching of DCF fluorescence (Pfaller et al. 2010), changing the color and increase the opalescence of experimental resin matrices (Yu et al. 2009), absorption and

scattering UV radiation as well as visible light (Wolf et al. 2001). NPs have also been shown to interfere with the comet assay by inducing additional breaks in the naked DNA during assay performance, induction of additional breaks from photocatalytic NPs in the nucleoid during performance of the assay, interference with scoring and interference with nucleoid DNA during electrophoresis (Karlsson et al. 2015).

1.2.2 Nanoparticles, Genotoxicity and Cancer

NPs can induce genotoxicity via primary (direct or indirect) or secondary mechanisms (Evans et al. 2017). Direct primary genotoxicity requires direct interaction of NPs with DNA or chromosomes potentially causing DNA lesions, mutagenesis, physical strand breakages or frameshift mutations (Schins and Knaapen 2007). During indirect primary genotoxicity the NPs does not interact directly with DNA but causes DNA damage through other molecules or mechanisms. The damage can be due to interactions with DNA repair proteins, disturbing cell cycle checkpoints, interactions with antioxidants and ROS generation (Magdolenova et al. 2014). There are tree general mechanisms in regards to oxidative stress: (i) Particles can by themselves generate oxidants which can cause DNA-damage. This will depend on the chemical and physical properties of different particles. Highly reactive hydroxyl radicals (OH•) can be created from transition metals via Fenton type reactions, for instance. (ii) Target cells can be stimulated by particles and produce genotoxic compounds or oxidants, which can induce cytochrome P450 enzymes or affect mitochondrial electron transport. (iii) Particles can cause inflammation where the inflammatory cells can produce oxidants (secondary genotoxicity) (Knaapen et al. 2004).

Secondary genotoxicity is caused by recruited immune cells such as macrophages and neutrophils, as a result of inflammation. These immune cells are recruited to clear the tissue from foreign particles which can result in an event known as oxidative burst where ROS is released (Evans et al. 2017). Cytokines and chemokines activate and recruits immune cells to sites of inflammation and are involved in the progression of inflammatory events (Feghali and Wright 1997). Generation of ROS can trigger release of pro-inflammatory cytokines via activation of redox sensitive signaling pathways such as MAPK and NF-κB which are in control of transcription of inflammatory genes e.g. IL-1β, IL-8, and TNF-α (Thannickal and Fanburg 2000). An overview of primary and secondary genotoxicity mechanisms are shown in figure 4.

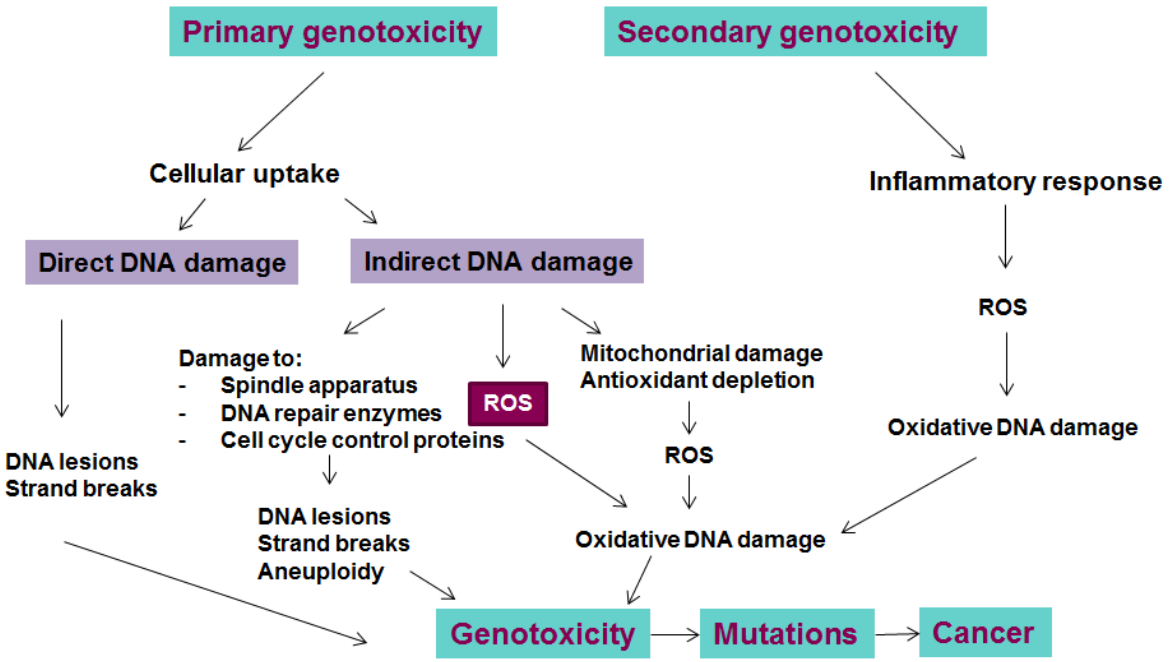

Figure 4. Overview of genotoxicity mechanisms induced by NPs.

Another mechanism that is discussed is that fact that NPs can be transported to the nucleus and hence be given direct access to the DNA. However this seems rather unlikely because the diameter of the nuclear pore complex is less than 8 nm (Terry et al. 2007). However, a few studies have found NPs in the nucleus, for instance titania (21 nm) and iron oxide (8.1 and 31.8 nm) NPs (Ahlinder et al. 2013), silver NPs (6-20 nm) (AshaRani et al. 2009) and silica NPs (40-70 nm) (Chen and von Mikecz 2005). A hypothesis for this phenomenon is that when the nuclear membrane breaks down during mitosis, particles may come in contact with DNA and this can lead to direct interactions. A consequence of this could be mechanical interference with the microtubules which could give rise to aneuploid cells during mitosis (Gonzalez et al. 2008). Intracellular metal ions from particles or particles themselves could also enhance the permeability of the lysosomal membrane. This can lead to DNase release into the cytoplasm where it could possibly pass into the nucleus and cut the DNA (Banasik et al. 2005).

1.3 NICKEL AND NICKEL OXIDE NANOPARTICLES

Ni NPs have unique characteristics that are only present at nano scale which includes high magnetism, a high level of surface energy, large surface area, low melting point, high and low burning point. Unfortunately, the same characteristics, in combination with their small size, cause the Ni NPs to pose a threat to human health. Studies have previously been conducted regarding Ni and genotoxicity. Common methods used have been micronucleus test, comet assay, Ames test and mammalian cell mutagenicity assays. However, the amounts of studies about Ni NPs and genotoxicity are still scarce, and most studies have been focusing on cytotoxicity. Non-genetic factors that may cause carcinogenicity that have been studied before in conjunction with Ni NPs are; enhanced oxidative stress, inflammatory response and abnormal apoptosis (Magaye and Zhao 2012).

1.3.1 Nickel Nanoparticles

A selection of studies regarding Ni NPs, of interest to the author, is presented in table 1.

Table 1. A selection of *in vitro* and *in vivo* involving Ni NPs.

Size (nm) **Concentration** **Time** **Organism**	**Results**	**Reference**
100 5, 10, 50, 100, and 200 µg/ml 24 h A549	Induction of higher levels of apoptosis than TiO_2 and silica fine particles. They also show that there was an increase in DNA fragmentation, about 20-24% and they hypothesize that these effects were due to generation of ROS.	(Park et al. 2007).
52.10 ± 6.40 2, 4, 8 & 20 µg/ml 24 & 48 h A431 cells	Cytotoxicity in a dose- and time-dependent manner. Oxidative stress evidenced by the generation of ROS and depletion of GSH. Dose- and time-dependent genotoxicity. Oxidative stress resulting in apoptosis and genotoxicity.	(Alarifi et al. 2014)
28 10, 25, and 50 µg/ml 24 h MCF-7 cells	Dose-dependent decreased cell viability and damaged cell membrane integrity. Oxidative stress induced in a dose-dependent manner. Dose-dependent induction of DNA damage. The level of mRNA and activity of caspase-3 enzyme was increased.	(Ahamed and Alhadlaq 2014)
<100 0-20 µg/cm^2 24, 48 & 72 h H460 cells	Dose-dependent and time-dependent toxicity, observed by reduced cell number. Ni NPs induced cleavage of caspase-3, caspase-7, and PARP in H460 cells within 48-72 h of exposure, indicating apoptosis.	(Pietruska et al. 2011)
<100 0.1, 1, 5, 10, 20 and 40 µg/cm^2 4, 24 and 48 h A549 cells	Increased cytotoxicity in the highest doses (20-40 µg cm^2). Increased CFE, suggesting higher proliferation, in low doses (0.1 or 1 µg cm^2).	(Latvala et al. 2016)
92.32 ± 29.69 0.5-20 µg/cm^2 1, 3, 6, 8 & 24 h JB6 cells	Higher AP-1 and NF-κB activation and a larger decrease of p53 transcription activity than fine particles. Induction of a higher protein level expressions for R-Ras, c-myc, C-Jun, p65, and p50 in a time-dependent manner. Increase anchorage-independent colony formation.	(Magaye et al. 2014a)
92.32 ± 29.69 0-20 µg/cm^2 1, 3, 6, 8 & 24 h JB6 cells	Induction of higher cytotoxicity and apoptosis than fine particles in JB6 cells. Apoptotic cell death through a caspase-8/AIF mediated cytochrome *c*-independent pathway.	(Zhao et al. 2009)
40.50 ± 18.6 0, 2.5, 5, 7.5 & 10 µg/cm^2 24 h JB6 cells	Dose-dependent cytotoxicity. EGCG caused a certain inhibition on toxicity. EGCG reduced apoptotic cell number and ROS. EGCG downregulated Ni NPs-induced activation of activator protein-1 and NF-κB. EGCG eased toxicity of Ni NPs through regulation of protein changes in MAPK signaling pathways.	(Gu et al. 2016)

62 0.5, 5, and 50 ug/ml 24 h HDMEC	Dose-dependent cytotoxicity. Difference in effects on oxidative stress and pro-inflammatory response. Different effects were observed when comparing to ions as well as indication of differences in the signaling pathways.	(Peters et al. 2007)
50 1 mg/kg, 10 mg/kg, and 20 mg/kg, intravenous injection 15 days Rat	Acute inflammation in lung, shown by lymphocytic infiltration in all dose groups and foamy macrophages in the high dose group. A high percentage of monocytes in the blood. Intravenous injection of Ni NPs may impact the liver, lung, spleen, and heart.	(Magaye et al. 2014b)
5 ($Ni(OH)_2$) NPs 500 $\mu g/m^3$ 5 h Mice	Acute inhalation exposure significantly increased both bone marrow EPCs and their levels in circulation. Indication of endothelial damage due to exposure. Tube formation and chemotaxis, but not proliferation, of bone marrow EPCs was afflicted. Decrease in mRNA of receptors involved in homing and mobilization of EPC.	(Liberda et al. 2014)
40.50 ± 18.6 0, 1, 5, 10, 15 and 25 $\mu g/cm^2$, cells 5.6, 12 and 25 mg/kg, intratracheal instllation A549 & Rat	Higher potency in cell toxicity and genotoxicity *in vitro* compared to Ni FPs. Ni NPs and FPs induced toxicity in organs of the SD rats and effects were similar for both particle types.	(Magaye et al. 2016)

In addition two case reports were found. A 38 year old healthy male was exposed to Ni by inhalation using a metal arc process while spraying Ni onto bushes for turbine bearings. He passed away 13 days later and adult respiratory distress syndrome was determined as the cause of death. The presence of Ni NPs where found in the lung but only in macrophages. The diameter of the NPs produced by the Ni metal arc process was on average about 50 nm while the particles in the macrophages were 25 nm or less in diameter. The particles appeared to exist in the lysosomes and could not be observed in other lung cells (Phillips et al. 2010). A female, 26 years old worked with Ni NP powder. The task included weighing out and handling on a lab bench without protective equipment. She developed nasal congestion, throat irritation, facial flushing, "post nasal drip" and new skin reactions to her belt buckle and earrings, which were temporally related to working with the NPs. She had a positive reaction to Ni on the T.R.U.E. patch test, and a normal range forced expiratory volume in one second that increased by 16% post bronchodilator. It was difficult for her to return to work, even in other parts of the building, because the symptoms reoccurred (Journeay and Goldman 2014).

1.3.2 Nickel Oxide Nanoparticles

A selection of studies regarding NiO NPs, of interest to the author, is presented in table 2.

Table 2. A selection of *in vitro* and *in vivo* involving NiO NPs.

Size (nm) **Concentration** **Time** **Organism**	**Results**	**Reference**
44 2-100 µg/mL 24 h HepG2 cells	Cytotoxicity (cell death) and dose dependent ROS. Vitamin C reduced cell death indicating that oxidative stress plays an important role. Micronuclei induction, chromatin condensation and DNA damage. Cell death could be induced via an apoptotic pathway.	(Ahamed et al. 2013)
15-24 25, 50 and 100 µg/mL 24 h HepG2 cells	Oxidative stress, DNA damage, apoptosis and transcriptome alterations	(Saquib et al. 2018)
15.0 ± 4.2-38.1 range of 0-500 µg/mL 24 h SH-SY5Y cells	Uptake in dose dependent manner. Morphological changes, dose-dependent DNA damage, apoptosis, oxidative damage.	(Abudayyak et al. 2017a)
4.2-38.1 0-500 µg/mL 24 h NRK-52E cells	Dose-dependent DNA damage and oxidative damage increasing levels of MDA, 8-OHdG, PC and depletion of GSH. Apoptotic/necrotic effects and morphological changes.	(Abudayyak et al. 2017b)
<50 0.1, 1, 5, 10, 20 and 40 µg cm^{-2} 4, 24 and 48 h A549 cells	Increased cytotoxicity in the highest doses. Increased CFE, suggesting higher proliferation, in low doses 0.1 or 1 µg cm^2. ROS and DNA damage.	(Latvala et al. 2016)
<50 5, 10, and 20 µg/cm^2 24 and 48 h BEAS-2B cells	Uptake by the cells and release of Ni^{2+}. Cytotoxicity by apoptosis. Repressed SIRT1 expression and activated p53 and Bax. Overexpression of SIRT1 attenuated NiO NPs-induced apoptosis via deacetylation p53.	(Duan et al. 2015)
22 1-100 µg/ml 24 h HEp-2 & MCF-7 cells	Cell viability was dose-dependent reduced. Induction of dose-dependent oxidative stress by depletion of glutathione, induction of ROS and lipid peroxidation. Induction of caspase-3 enzyme activity and DNA fragmentation, biomarkers of apoptosis.	(Siddiqui et al. 2012)
<100 0-20 µg/cm^2 24, 48 & 72 h H460 cells	Dose-dependent and time-dependent toxicity by reduced cell number. NiO NPs induced cleavage of caspase-3, caspase-7 and PARP which indicates apoptosis.	(Pietruska et al. 2011)

20 0.015, 0.06, and 0.24 mg/kg, intratracheal instillation 6 w Rat	TGF-β1 content was increased. Upregulation of gene expression of TGF-β1, Smad2, Smad4, matrix metalloproteinase, and tissue inhibitor of metalloproteinase. Induction of pulmonary fibrosis, which may be related to activation of TGF-β1.	(Chang et al. 2017b)
19 0.2 and 1.0 mg, intratracheal instillation 0.32 and 1.65 mg/m³, inhalation 4 w Rat	NPs persisted for longer in the lung and biological half times was longer compared with TiO_2 NPs. Biopersistence correlated with inflammatory response, histopathological changes, and other biomarkers in BALF.	(Oyabu et al. 2017)
15-35 0.2 mg or 1.0 mg, intratracheal instillation 1.65 ± 0.20 mg/m^3 and 0.32 ± 0.07 mg/m^3, inhalation 4 w Rat	Pulmonary oxidative stress was induced by both administration methods. Single intratracheal instillation induced major pulmonary oxidative stress while inhalation induced milder and continuous oxidative stress.	(Horie et al. 2016)
30 and 100 0.2 mg (0.8 mg/kg) or 1 mg (4 mg/kg), intratracheal instillation 1.65 ± 0.20 mg/m^3, inhalation 4 w Rat	The inhalation of NiO induced neutrophil inflammation and related cytokine upregulation. The intratracheal instillation of NiO induced persistent and transient inflammation and upregulation of cytokines.	(Morimoto et al. 2016)
<50 1, 2, 4 mg/kg b.w/day, orally 1 & 2 w Rat	Increase in chromosomal aberrations, formation of micronuclei and DNA damage. Apoptosis, generation of ROS and dysfunction of mitochondrial membrane potential. Imbalance of antioxidant enzymes and histological alterations was observed in the liver.	(Saquib et al. 2017)
15.62 ± 2.59 125, 250 and 500 mg/kg bw, orally 18 & 24 h Rat	DNA damage and chromosomal changes at 500 mg/kg bw dose in the PBL, liver and kidney cells. Hepatic damage and mild alterations in kidneys.	(Dumala et al. 2017)
20 2 mg/kg bw, intratracheal instllation 3, 28 & 91 days Rat	BALF analyses revealed pulmonary injury, inflammation. Histopathological analyses demonstrated inflammatory response, phagocytosis of NiO by alveolar macrophages, degeneration and necrosis of alveolar macrophages.	(Senoh et al. 2017)
20 0.015, 0.06, and 0.24 mg/kg, intratracheal instllation 6 w Rat	Pulmonary fibrosis was induced and could be related to TGF-β1 activation. TGF-β1 content was increased, and upregulation in expression of the genes TGF-β1, Smad2, Smad4, matrix metalloproteinase and tissue inhibitor of metalloproteinase.	(Chang et al. 2017b)

18.6 ± 5.5 3.3 mg/kg, intratracheal instllation 3,7 & 28 days Rat & RAW264.7 cells	NLRP3 was upregulated, overexpression of active form of caspase-1 (p20) and IL-1β secretion *in vivo*. siRNA-mediated NLRP3 knockdown completely attenuated NiO NP induced cytokine release and caspase-1 activity in macrophages *in vitro*. Induction of NLRP3 inflammasome activation requires particle uptake and ROS production.	(Cao et al. 2016)
~20 0.015, 0.06, and 0.24 mg/ kg, intratracheal instllation 6 w Rat	Abnormal changes on indicators of nitrative stress, inflammatory cytokines and cytokine-induced neutrophil chemoattractants in lung tissue. Upregulated mRNA and protein expression of NF-κB, inhibitor of κB kinase-α and nuclear factor-inducing kinase.	(Chang et al. 2017a)
- 0. 015, 0. 06 and 0. 24 mg/kg 6 w Rat	Histopathology showed that the widened alveolar speta, inflammatory infiltration and NP deposition increased with the increasing dosage. Higher levels of IL-2, TGF-β and IFN-γ. Level of 8-OHd G increased in serum.	(Liu et al. 2016)
<50 800 μg/rat, intratracheal instllation 28 & 60 days Rat & human fetal lung fibroblasts	Pulmonary fibrosis *in vivo* and *in vitro*. TGF-β1 facilitated HIF-1α signaling by accumulating HIF-1α protein and enhancing DNA binding activity of HIF-1α. Activated HIF-1α promoted TGF-β1 expression in mRNA and protein level.	(Qian et al. 2015)

2 AIM

The overall aim of this thesis is to increase the knowledge about the mechanisms underlying the carcinogenicity of Ni and Ni compounds and particularly to elucidate if Ni in NP-form (Ni and NiO) act via different mechanisms compared to soluble Ni ($NiCl_2$), figure 5. The specific aims of the included studies were:

- To investigate genotoxicity and mutagenicity of Ni and NiO NPs compared to Ni ions/complexes from $NiCl_2$ using different methods and *in vitro* models (study I).

- To investigate the ability of Ni and NiO NPs to alter genome stability in human bronchial epithelial BEAS-2B cells and to discern possible mechanisms (study II)

- To investigate inflammation and genotoxicity caused by Ni and NiO NPs as well as to explore the possibility to test secondary (inflammation-driven) genotoxicity in vitro (study III)

- To investigate Ni-induced cellular changes of relevance for cancer with a focus on epithelial to mesenchymal transition and a stem cell like phenotype (study IV)

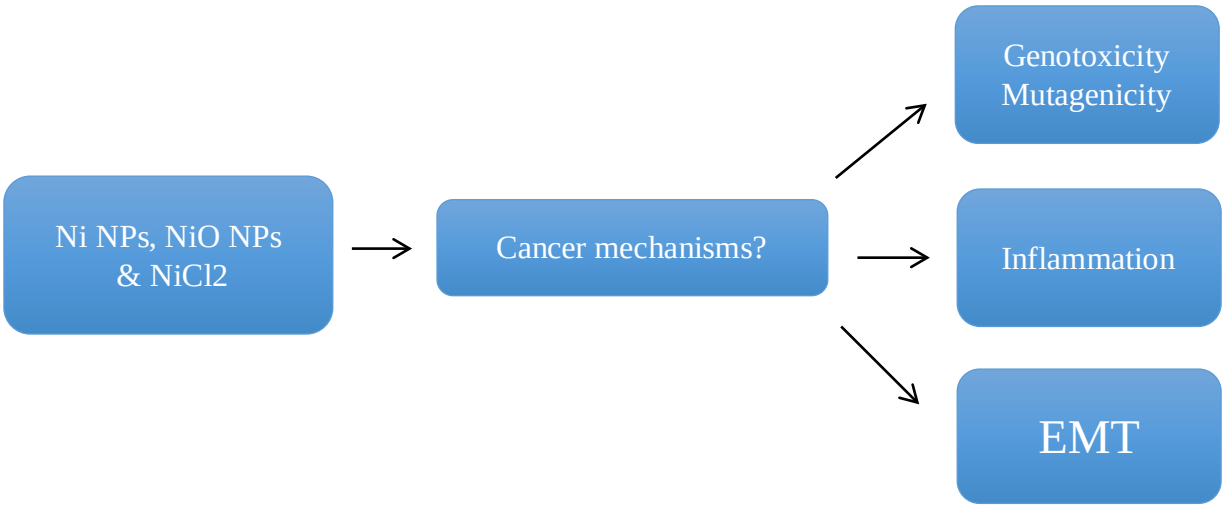

Figure 5. Overview of the aims of the studies.

3 METHODS

In this chapter the principals of the methods used in this thesis will be described. Detailed technical information can be found in the materials and methods section for each associated study.

3.1 NANOMATERIALS

Table 3. Nanoparticles used in this thesis.

NP	BET-area[a] (m^2/g)	Primary size[b] (nm)
Ni	6.41	<100
NiO	102	<50

[a] Data reported in Latvala et al. (2016).

[b] TEM images shown in Di Bucchianico et al. (2018), Kain et al. (2012) and Latvala et al. (2016).

3.2 CELL MODELS

The cell line HBEC3-KT, used in study I and III, are normal human bronchial epithelial cells, immortalized by transfection with a retroviral construct containing cyclin-dependent kinase 4 and human telomerase reverse transcriptase. They do not form tumours in mice or colonies in soft agar (Ramirez et al. 2004). Furthermore, full malignant phenotype could not be confirmed after inducing multiple oncogenic changes (*K-RAS*^{V12}, p53 knockdown, *mutant EGFRs*) (Sato et al. 2006). Hence HBEC3-KT are suitable to use as an *in vitro* model to mimic "normal lung cells" as they are considered to display a non-cancerous phenotype.

The cell line BEAS-2B, used in study II and IV, are human bronchial epithelial cells obtained from non-cancerous individuals and immortalized with 12-SV40 virus (Reddel 1989). They do not form tumours in mice but they can undergo squamous differentiation in the presence of serum (Lechner et al. 1984; Reddel 1989). BEAS-2B has been suggested to be an appropriate *in vitro* model to study heavy metal induced carcinogenesis (Park et al. 2015) and it has been used to study malignant transformation after exposure to chromium and arsenite (He et al. 2013; Zhang et al. 2012).

The cancerous cell line THP-1, used in study III, are human monocyte cells originally obtained from a 1 year old infant with acute monocytic leukemia (Tsuchiya et al. 1980). THP-1 monocytes can be differentiated into macrophages with the ester phorbol 12-myristate 13-acetate (PMA) and acquire phenotypic as well as functional characteristics reassembling

primary macrophages. After differentiation the cells stop proliferating, change morphology and become adherent to the cell culture plastic (Auwerx 1991).

3.3 CHARACTERIZATION OF NANOMATERIALS

3.3.1 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS), used in study II, is a spectroscopic technique that can be used to analyze the surface chemistry of a material. Elemental composition, chemical state, empirical formula and electronic state of the elements within a material can be measured using XPS. A surface is irradiated by a beam of X-rays while the kinetic energy is measured simultaneously and emitted electrons from the 1-10 nm top of the material are analyzed which results in a XPS spectra. Electrons over a range of electron kinetic energies are counted and a photoelectron spectrum is then recorded. Atoms which emits electrons of a particular characteristic energy generates peaks in a spectrum. Identification and quantification of surface elements can be interpreted from energies and intensities of the photoelectron peaks.

3.3.2 Photon Cross-correlation Spectroscopy

Photon cross-correlation spectroscopy (PCCS), used in study I and II is used to measure particle size distribution. In PCCS the sample is illuminated by two beams, at different azimuthal angles, which were split from the initial light source. Two detectors are used to collect the scattered light, collected at two scattering planes, with exactly the same scattering vector and the light intensities are cross-correlated. Multiple scattered lights, has no cross-correlation hence it is filtered out and remaining is the single scattering. The two lights have to match precisely otherwise they will not be correlated (Xu 2008).

3.3.3 Transmission Electron Microscopy

Transmission electron microscopy (TEM), used in study II, is often used to visualize intracellular location of nanomaterials as well as information about their size, shape or aggregation state. A beam of electrons is transmitted through the sample and then forms an image on a screen. The resolution in TEM is significantly higher than in a regular light microscope which enables detection of even single column atoms (Asadabad and Eskandari 2015).

3.3.4 Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS), used in study I, II and III, can detect metals at low concentrations. The sample is ionized by inductively coupled plasma and then the mass spectrometer is used to separate and quantify the ions. It can be used to study release of metal from for instance NPs in cell media and hence give information on the stability of the particles or release of ions (Laborda et al. 2016).

3.4 CELL VIABILITY – ALAMAR BLUE ASSAY

Alamar blue assay, used in study I, III and IV, is one of the most referenced assays used for cell viability and cytotoxicity and has been used for 50 years in biological as well as environmental studies. The assay monitors the reducing environment of living cells where the active ingredient is resazurin which is a blue non-fluorescent dye that is reduced to the highly fluorescent pink resorufin by metabolically active cells. The dye accepts electrons in the electron transport chain and then changes color, without interfering with the normal transfer of electrons. Fluorescent signals can then be measured at excitation wavelength 530-560 nm and emission wavelength 590 nm. The advantages with the assay are; high sensitivity and linearity, provides accurate time-course measurements, ideal for the use with post-measurement functional assays, involves no cell lysis, scalable and can be used with absorbance- and/or fluorescence-based instrumentation platforms, flexible because it can be used with different cell models and finally, non-toxic as well as non-radioactive and safe for the user, as well as the environment (Rampersad 2012).

3.5 EVALUATION OF ROS GENERATION

The H_2DCF-DA (dihydrodichlorofluorescein diacetate) assay, used in study I, II and III, is commonly used to detect intracellular ROS. H_2DCF-DA is distributed to the cells and oxidation of the compound to DCF is measured over time. H_2DCF-DA is non-fluorescent in its form but since it is a lipophilic ester it can easily cross the plasma membrane and enter the cytosol where it is cleaved to DCF by unspecific esterases. DCF is a fluorochrome that after excitation with blue light, at around 488 nm, emits green light, around 525 nm, which can be measured using a plate reader. The measured brightness of the fluorescence is supposed to reflect the presence of ROS but cannot tell which type of ROS (Halliwell and Whiteman 2004).

3.6 FLOW CYTOMETRY

Flow cytometry, used in study I, II and IV, can be used to measure physical and chemical characteristics of a cell or particle population. The technique offers quick analysis of thousands of cells/particles per second as well as multi-parameter analysis of single cells. Four main components are found inside the instrument; fluidics (generating a sheath fluid consisting of single cells), excitation optics (consisting of multiple lasers), collection optics (transmitting the emitted wavelengths to the photomultiplier tube/detector arrays in multiple angles) and analyzer (transforming electrical signals to digital signals and displaying quantitative and qualitative data). The cells/particles in suspension are directed to go through a laser beam, one cell at the time hydrodynamically focused in a stream, and the light is scattered and absorbed by detectors and signals are processed and digitized for analysis by a computer. The cells are usually stained with fluorescent markers such as antibodies conjugated with a flourochrome which can be excited by the laser beam. The excited energy is released as a light photon with specific spectral properties that are different for different fluorochromes. Several detectors are aimed at the point where light beam and stream meet to detect forward scatter (FSC), side scatter (SSC) as well as fluorescence. FSC can be used to

determine size and SSC for granularity/complexity of the cell/particle. Flow cytometry are used in research, clinical trials and clinical practice and can be used for detection of; cytoplasmic complexity/granularity, nucleic acid content, various intracellular/membrane proteins, cell counting, cell size and volume, expression of cell surface and intracellular molecules, purity of isolated subpopulations as well as diagnosis of health disorders and sorting of cells (Woo et al. 2014).

3.7 TOXTRACKER

The Toxtracker genotoxicity assay, used in study I, is based on six mouse embryonic reporter cell lines (mES cells). mES cells in comparison to cancer cell lines are genetically stable and proficient in all cellular pathways required for accurate detection of potentially carcinogenic properties of compounds. Each cell line is transfected with the reporter gene GFP (green fluorescent protein) which have been inserted in the promoter region of the gene of interest. This assay contains a panel of six reporters marking genes of interest for different endpoints considered important in carcinogenesis; Bscl2 and Rtkn: DNA damage, Srxn1 and Blvrb: oxitative stress, Ddit3: protein damage and Btg2: cellular stress. When these transfected cells are exposed to a chemical/xenobiotic that will activate transcription of the gene of interest they will exhibit fluorescence. The fluorescence is detected using flow cytometry and the cells are usually seeded in a 96 well plate which allows for higher throughput. This panel of genes was selected by exposure to over 40 different carcinogenic chemicals following whole-genome transcription profiling (Hendriks et al. 2016).

3.8 *HPRT*-MUTATION ASSAY

The hypoxanthine phosphorybosyl transferase (*HPRT*) gene, situated on the X-chromosome, is used as a model to detect mutations in mammalian cell lines after exposure to chemicals. The assay, used in study I, is included in guidelines for mammalian gene mutation tests OECD (1997), Organisation for Economic Co-operation and Development. After exposure and fixation of the mutation in the cells the compound 6-thioguanine is added. 6-thioguanine, an analogue to the purine base guanine, will incorporate itself in the DNA and terminate the cells which did not gain mutation in the *HPRT*-gene. The cells which acquired mutation in the *HPRT*-gene will survive and present as colonies. Colony forming efficiency (CFE) are used to measure cell viability after initial exposure and before exposure to 6-tioguanine. The assay responds to a broad spectrum of mutagens unlike other bacterial assays (Johnson 2012). figure 6 shows a schematic presentation of the steps included in the assay.

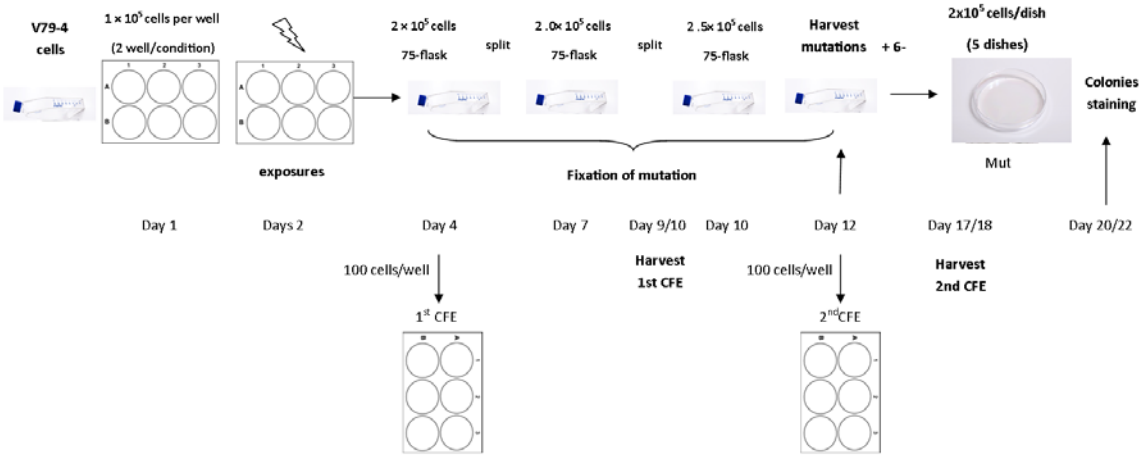

Figure 6. Schematic overview of the *HPRT*-mutation assay.

3.9 COMET ASSAY

The comet assay, used in study I, II and III, also referred to as "single-cell gel electrophoresis", is most widely used in the alkaline version, where using a pH over 13 enables the detection of single-strand breaks in the DNA. The single-strand breaks can be associated with alkali-labile sites and incomplete excision repair. In short, cells are (after exposure) harvested, embedded in agarose-gel on a microscope slide and lysed. The lysis solution contains Triton X-100, which breaks down the membranes, and also NaCl in a high concentration that removes histones and other soluble proteins. The supercoiled DNA attaches to a nuclear matrix and a structure named "nucleoid" is created. The DNA is then unwound by incubation in alkaline electrophoresis buffer and the electrophoresis is further executed under the same conditions. The damaged DNA is negatively charged and migrates towards the anode during the electrophoresis which results in an appearance of comet-like structure with a head and a tail. The next steps are neutralization and fixation following staining of the DNA. The comets can then be visually analyzed using a fluorescent microscope with the help of a software specific for the assay. Different parameters for each comet are calculated and the most commonly used ones are; tail length, percentage DNA in the tail and tail moment (tail length × the total tail intensity). Percentage tail DNA is generally regarded as more useful and easier to interpret, and therefore recommended to use.

Oxidatively damaged DNA can also be detected by a modification of the assay. A lesion specific endonuclease is added following the lysis. This will remove the damaged base and create an abasic site that becomes transformed into a strand break by the lyase activity of the enzyme, or by the alkaline treatment step. One of the common enzymes used are formamidopyrimidine DNA glycosylase (FPG) that recognize oxidatively damaged purines mainly formamidopyrimidines and 8-oxoguanine (Karlsson et al. 2015).

3.10 MICRONUCLEUS ASSAY

The micronucleus assay, used in study II, can be used for evaluation of cytogenetic damage after exposure to chemicals. When inclusion of acentric or whole chromosomes in the daughter nuclei fails, micronuclei are formed. This occurs because they cannot attach to the mitotic spindle and migrate to the poles in the anaphase in the cell division. Reasons behind formation of micronuclei can be; defects in the kinetochore assembly, unresolved replication stress, dysfunctional spindle or unrepaired double strand breaks. Cytochalasin B, added to cells after exposure, inhibits cytokinesis and enables distinction of cells that underwent cell division, which will appear binucleated. This enables for scoring of only binucleated cells and the confounding effects of impaired kinetics of cell division are reduced. In this way, only cells that were damaged during exposure are scored. Cells are allowed to grow for a period of time sufficient for chromosomal damage to occur leading to the formation of micronuclei in bi-or multinucleated cells in interphase. The cells are then harvested and stained and analyzed by microscope for the presence of micronuclei (Fenech 2007; Fenech et al. 2011)

3.11 PROTEOME PROFILER ANTIBODY ARRAY

Proteome Profiler™ Antibody Arrays, used in study IV, are commercially available kits from R&D Systems that allows for parallel detection of the relative levels of a selection of proteins. The technique is a membrane-based sandwich immunoassay. Proteins in the sample bind to capture antibodies on a membrane and biotinylated detection antibodies in turn bind to the proteins. The results are visualized with chemiluminescent detection reagents and the signal is proportional to the bound analyte (figure 7). The Proteome Profiler Human XL Cytokine Array Kit can detect 105 different cytokines. The Proteome Profiler Human Chemokine Array Kit can detect 31 different chemokines.

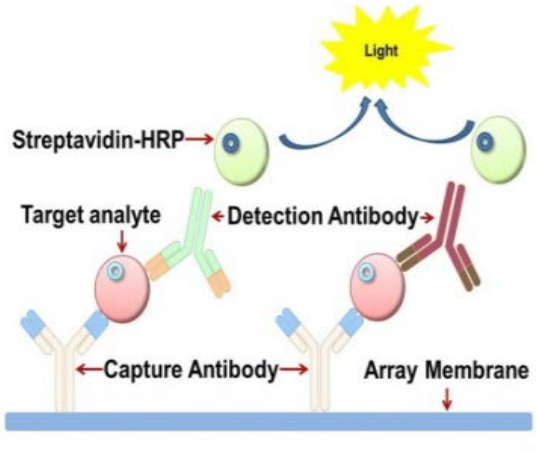

Figure 7. Principal of the Proteome Profiler™ Antibody Arrays.

3.12 MULITPLEX ASSAY

The purpose of multiplex arrays, used in study III, is to measure multiple cytokines at the same time from the same sample. The most common detection method is by flow cytometry, which is based on beads. The beads are coated with specific capture antibodies which the proteins in the sample bind to. Detection antibodies conjugated with a flourochrome binds to the protein and creates a cytokine-capture antibody complex on the bead set, similar to figure 7. Multiple cytokines in a liquid sample can thus be recognized using a setup of different bead sets (Leng et al. 2008).

3.13 IMMUNOFLUORESCENCE

Immunofluorescence, used in study IV, is a method that gives information about the structure of the cell and the locations of specific molecules. The cell or tissue of investigation is incubated with an antibody specific to the target of interest. A secondary antibody is then added conjugated to a fluorophore which makes it possible to detect the signal by a fluorescent microscope (Donaldson 2015).

3.14 IN-CELL WESTERN

In-cell western, used in study IV, is a quantitative immunofluorescence assay used to measure specific proteins inside or on cells. It can be described as a combination of western blot and ELISA. Cells are cultured in a 96- or 384-well plate, fixed, permeabilized, blocked for unspecific binding and incubated with a primary antibody. The next steps are washing and incubation with a secondary antibody which is conjugated to an infrared dye. The infrared dye is excited with a laser and emits fluorescence which is detected by a scanner, which reads the culture plate. The scanner can detect two targets in the same sample as it has two detection channels. This allows for normalization to the cell number as the cells can be stained with a cell-tag infrared dye. Detection of proteins in their cellular context increases the precision of quantification.

3.15 REAL-TIME PCR

Real-time polymerase chain reaction (PCR), used in study IV, is based on the conventional PCR but the difference is that the reaction is monitored in real-time. The products generated during each cycle of the PCR process are detected and measured and are directly proportional to the starting amount of template. The readout is a fluorescent signal and measurement is registered after each round of amplification and the results are presented in an amplification plot that takes the shape of a linear sigmoid curve. If the amplification is 100% efficient it results in doubling of the PCR product in each cycle and this occurs in the exponential phase. The fractional PCR cycle number where the reporter fluorescence is greater than the minimal detection level is called the threshold cycle (C_T) and it represents the parameter used for quantification and it is used to compare samples (Arya et al. 2005).

3.16 THE SCRATCH WOUND HEALING ASSAY

The purpose of the scratch wound healing assay, used in study IV, is to study cell migration in vitro. The method is fairly simple and the principal is based on a creation of an artificial scratch in the confluent cell monolayer. The cells in the edge on the scratch will then move and try to close the scratch. This process is monitored by taking pictures just after the scratch is created, and with regular time intervals and then comparison of the pictures will be performed to determine the cell migration rate (Liang et al. 2007).

3.17 INVASION/MIGRATION ASSAY

The transwell invasion/migration assay, used in study IV, is commonly used in cancer research and can be used to study if cells can migrate spontaneously or migrate toward a chemoattractant. The cells are plated in the top well of one setup of transwells containing appropriately sized pores for studying migration and another setup of transwells containing matrigel for invasion. The chemoattractant are placed in the bottom well. The cells are then allowed to migrate or invade through the top well and these cells are then stained and counted in a microscope. The results can be presented as either the number of migrating and invading cells separately or by calculating the invasion index by the ratio of invading/migrating cells (Justus et al. 2014).

4 RESULTS

4.1 STUDY I: GENOTOXIC AND MUTAGENIC PROPERTIES OF NI AND NIO NANOPARTICLES INVESTIGATED BY COMET ASSAY, Γ-H2AX STAINING, HPRT MUTATION ASSAY AND TOXTRACKER REPORTER CELL LINES

The aim of this study was to study genotoxicity of Ni and NiO NPs in comparison to Ni ions/complexes from soluble $NiCl_2$ using different model systems.

Cytotoxicity was measured and Ni and NiO NPs were found to be non-cytotoxic at the doses tested while $NiCl_2$ caused a slight decrease in viability at the highest dose. Next, the comet assay was performed and Ni and NiO NPs induced DNA strand breaks at the doses tested (5-25 μg/mL) while $NiCl_2$ did not. Thereafter, γ-H2AX staining using flow cytometry was conducted to investigate the formation of double strand breaks. The results showed no significant induction of γ-H2AX foci in neither of the doses tested (5-25 μg/mL). The exposure time was 24 h for all the experiments.

The DCFH-DA assay was employed to investigate intracellular ROS generation. A statistically significant increase was observed for Ni and NiO NPs at the higher doses (25 and 50 μg/mL), while no significant changes could be observed for $NiCl_2$.

Six different reporter cell lines (ToxTracker) were used to investigate the underlying mechanisms of the genotoxicity of Ni and NiO NPs. Cell viability was initially measured with the aim to determine test concentrations reaching up to approx. 50% cytotoxicity and large differences between Ni NPs, NiO NPs and $NiCl_2$ was observed. Ni NPs could further only be tested at doses up to 5 μg/mL because they were found considerably more cytotoxic than NiO NPs and $NiCl_2$. The NiO NPs and Ni ions were tested at doses up to 100 μg/mL. The oxidative stress reporter *Srxn1* was triggered by all compounds tested, where an increase was found at low doses (below 1 μg/mL) for Ni NPs, at higher doses (5-10 μg/mL) for NiO NPs and even higher doses (30-50 μg/mL) for $NiCl_2$. The *Bscl2* reporter for stalled replication forks was not induced by any of the exposures. *Rtkn*, the reporter for NFκB signaling, showed a small increase for all the three compounds. The reporter for protein stress, *Ddit3,* was induced at the highest doses for all the three compounds.

The *Hprt* gene mutation assay in mES cells and V79-4 cells was employed to test mutagenicity of Ni NPs, NiO NPs and $NiCl_2$. There was a slight increase in the mutant frequency in the mES cells for some of the Ni, NiO and $NiCl_2$-exposures but statistical significance was only found for one dose (0.5 μg/mL) of NiO. Due to large variation between the experiments no statistically significant increase in mutation frequency could be observed for the V79-4 cells. The results from this study are concluded in table 5.

Table 5. Concluding table of the results presented in study I.

	Ni	**NiO**	**$NiCl_2$**
Cytotoxicity HBEC ≤50 µg/mL	No	No	Minor at 50 µg/mL
DNA strand breaks HBEC ≤25 µg/mL	Yes from 10 µg/mL	Yes from 5 µg/mL	No
DNA double strand breaks HBEC ≤25 µg/mL	No	No	No
Cell-free ROS	Minor	Yes	Minor
Intracellular ROS HBEC ≤50 µg/mL	Minor	Yes	No
Cytotoxicity reporter cells	Yes	Yes	Yes
Oxidative stress reporter induction	Yes	Yes	Yes
DNA damage reporter induction	No[1]	No[1]	No[1]
Protein stress reporter induction	Yes, at high cytotoxicity	Yes, at high cytotoxicity	Yes at high cytotoxicity
***Hprt* mutations mES cells ≤5 µg/mL**	No	At one dose	No

[1]A modest increase in *Rtkn* reporter but not reaching the ×2 threshold

4.2 STUDY II: CALCIUM-DEPENDENT CYTO- AND GENOTOXICITY OF NICKEL METAL AND NICKEL OXIDE NANOPARTICLES IN HUMAN LUNG CELLS

The aim of the study was to in depth study the ability of well characterized Ni and NiO NPs to alter genome stability in comparison to Ni ions/complexes from soluble $NiCl_2$ and to investigate underlying mechanisms in BEAS-2B cells.

Characterization of the NPs was initially performed. TEM analysis showed a variation in size but in general Ni NPs were less than 100 nm and NiO NPs less than 50 nm. The intrinsic ROS generating ability was analyzed by the DCFH assay. NiO NPs was highly reactive in the absence of HRP whereas Ni and NiO showed similar but smaller effects in the presence of HRP. No effects were observed for $NiCl_2$.

The hydrodynamic size and light scattering using PCCS as well as the dissolution/Ni release by means of ICP-MS was performed to characterize the behavior of the NPs in cell medium. Directly after dispersion (0 h), agglomeration of Ni and NiO NPs in cell medium occurred with an average size around 500 nm for the Ni NPs and 750 nm for NiO NPs. Measurements after 2 and 24 h showed similar characteristic as the cell medium, which indicates that the particles was removed from the solution by sedimentation. Release of Ni in solution was increased with time and around 5% and 9 % Ni release was observed after 48 h for Ni and NiO NPs, respectively, at the highest concentration. No significant differences could be found between Ni and NiO NPs at the lower concentrations (1 and 5 μg Ni/mL) after 48 h. In regards to uptake, TEM was applied and the results showed that both Ni and NiO NPs were taken up by BEAS-2B cells. The cellular content of Ni after 48 h exposure was also measured with ICP-MS, where uptake was observed for Ni (40 μg) and NiO (60 μg) measured per million cells, whereas there was no significant uptake of $NiCl_2$.

Annexin V/PI staining was performed to measure apoptosis and necrosis after $NiCl_2$, Ni and NiO NP exposures at concentrations of 1, 5 and 10 μg Ni/ml. There was a dose dependent increase in apoptosis for Ni and NiO NPs whereas NiO was the most potent. Regarding necrosis, an increase by concentration was also observed where the effect was apparent particularly at the highest concentrations (10 μg Ni/mL) of NiO NPs and $NiCl_2$. The same trend was also observed in the CBMN Cyt assay. The replication index was increased by the lowest concentration of Ni NPs tested while the highest concentration of NiO NPs and $NiCl_2$ showed a significant cytostatic effect and a reduction of the mitotic index.

The comet assay was employed for investigation of DNA strand breaks and the results showed a significant increase in all concentrations tested (1, 5 and 10 μg/mL) for all three compounds, however NiO NPs was found to be most potent causing a threefold increase. Intracellular ROS was also measured with the DCFH-DA assay and a significant increase was observed in response to NiO NPs (1, 5 and 10 μg/mL) as well as $NiCl_2$ (5 and 10 μg/mL). Intracellular calcium levels were measured using the fluorescent probe Fluo-4, with an exposure of 5 μg/mL. A significant increase was observed for NiO NPs and $NiCl_2$, while higher variation among experiments led to a non-statistically significant increase for Ni NPs.

The frequency of micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were analyzed. A significant increase in MN binucleated cells could be observed for all the exposures at the two highest concentrations, where NiO NPs were found to be most potent. Ni NPs and $NiCl_2$ were more potent than NiO NPs in inducing MN in mononucleated cells, NBPs as well as NBUDs. The effects on calcium and iron were evaluated using the same assay in combination with various chelators and inhibitors. Cells were co-exposed to calcium modulators or one iron modulator and the different Ni exposures ($NiCl_2$, Ni and NiO NPs) in concentration of 5 µg Ni/mL. A significant reduction in MN induced by NiO NPs and $NiCl_2$ was observed after co-exposure with the iron chelator deferoxamine while Ni NPs showed a smaller difference. Similar results were seen for NPB and NBUD. After co-exposure to the calcium modulators BAPTA-AM (Ca^{2+} chelator) and verapamil (inhibits calcium uptake through the plasma membrane) the genotoxicity was reverted to control values for all the Ni exposures. Co-exposure with dantrolene (prevention of Ca^{2+} release from the endoplasmic reticulum) was protective against genotoxicity induced by Ni and NiO NPs but not by $NiCl_2$. Similarly, protective effects were also shown for apoptosis and necrosis induced by NiO NPs. A summary of the results are presented in figure 8.

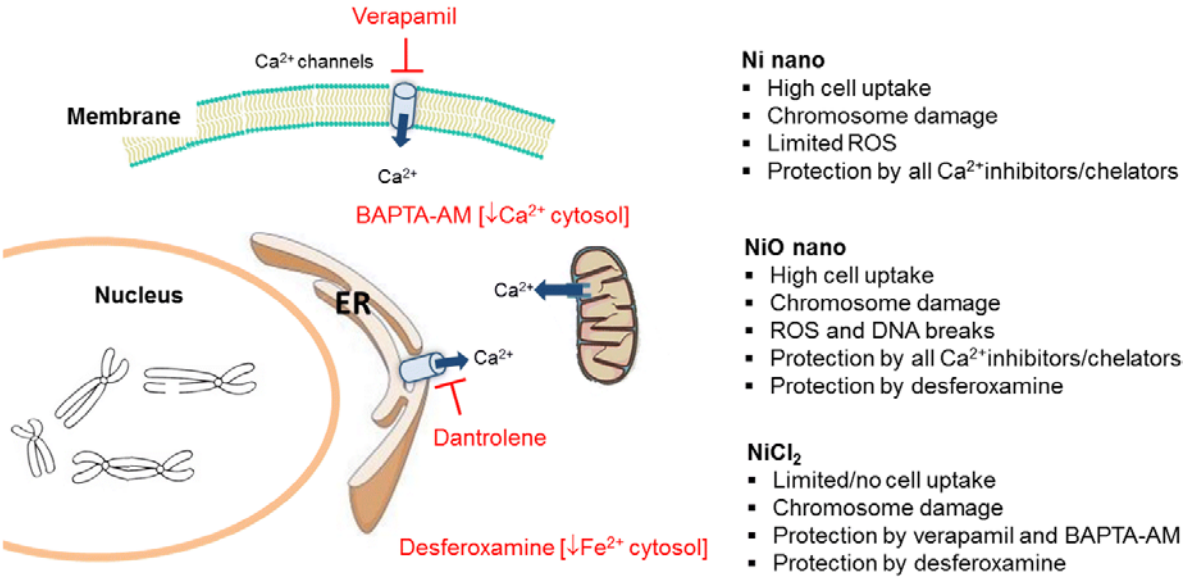

Figure 8. Summary of the results presented in study II.

4.3 STUDY III: INFLAMMATION AND (SECONDARY) GENOTOXICITY OF NI AND NIO NANOPARTICLES

To this date, few studies have been performed on the secondary genotoxicity of NPs *in vitro*. The aim of this study was to test the hypothesis that Ni and NiO NPs can cause both primary and secondary genotoxicity *in vitro*.

The cytotoxicity was measured after exposure to Ni and NiO NPs at concentrations of 5, 10, 25 and 50 µg/ml for 3 and 24 h in THP-1 derived macrophages (THP-1*) and HBEC cells. No cytotoxic effect was observed in response to Ni and NiO NPs in HBEC cells while in THP-1* a decrease in cell viability could be observed in the highest concentrations after 24 h.

The proteome profiler antibody array was used to investigate release of cytokines from THP-1* cells exposed to 50 µg/ml Ni and NiO NPs, after 3 and 18 h exposure. 105 different cytokines and 31 chemokines (only used after 3 h exposure) were analyzed. Quartz (SiO_2) was included as a benchmark particle for comparison. The release of the following cytokines was increased after 3 h exposure; MIF, IFN-γ, IGFBP-3, IL-17A and IL-12p70 (based on two times cut-off). No increased release was observed for the chemokines except from ENA-78 (NiO NPs exposure). The release of the following cytokines was increased after 18 h exposure; CD40 Ligand, C-Reactive Protein, Fas Ligand, FGF basic, Flt-3 Ligand, IL-1α, IL-1β, VEGF and G-CSF. In general, the trend was similar for Ni and NiO as well as the SiO_2 benchmark particle. VEGF was an exception where an increase was seen for Ni and NiO but not for SiO_2. Furthermore, some cytokines were specific for SiO_2 including IL-17A, IP10 and MCP1. VEGF and IL1β were also measured by Luminex. Ni and NiO, but not SiO_2, caused an increase of VEGF after 18 h exposure. A small, non-statistically significant increase for IL-1β was observed after 18 h.

Thereafter we wanted to investigate if secreted factors from the exposed macrophages (50 µg/ml of Ni, NiO NPs and SiO_2) could induce DNA damage in HBEC cells. HBEC cells were exposed to conditioned media (CM), figure 9A, generated from the 3 and 18 h exposed macrophages, and further investigated by the comet assay. The genotoxic effect directly caused by Ni, NiO NPs and SiO_2 was also tested (10 µg/ml, 3 h) as well as the combined exposure NPs+CM. The CM, NPs as well as the combination of CM and NPs induced statistically significant DNA damage compared to control (2.4% DNA in tail). CM from Ni and NiO NPs caused 7.3% and 8.7% DNA in tail, respectively, which is comparable to the damage induced directly by the NPs (10 µg/ml), being 9.2% and 11.6% DNA in tail for Ni and NiO NPs, respectively. The damage was also comparable to the combined exposure from NPs+CM, hence no additive effect was observed.

The cells were also cultured in a transwell co-culture system, figure 9B, with THP-1* in the top well and HBEC cells in the bottom well. The THP-1* cells were exposed to 50 µg/ml of Ni, NiO and SiO_2 NPs for 3 and 18 h. The HBEC cells were then further analyzed by comet assay. Exposure to Ni and NiO NPs resulted in a significant increase in DNA strand breaks

compared to control while SiO_2 only showed a small non-significant increase, for both 3 and 18 h.

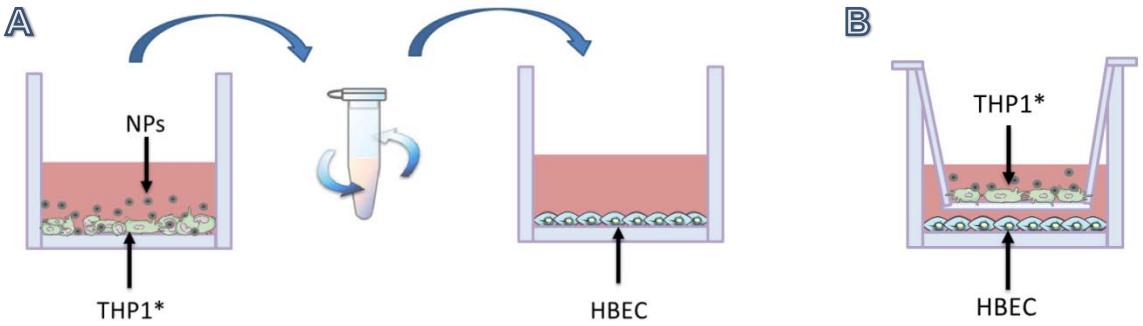

Figure 9. Descriptive figure of conditioned media (A) and co-culture system (B).

We investigated the amount of released Ni present in CM generated from Ni and NiO NP exposed THP-1* for 3 h and 18 h, and compared it to the total amount of added Ni and NiO NPs (50 µg/ml). The percentage of Ni released from Ni and NiO NPs were 1.4% and 2.3% after 3 h, respectively as well as 2.3% and 5.5%, respectively, after 18 h. We also used the DCFH-DA assay to measure ROS, however we were not able to measure any increased ROS in the CM but an increase in intracellular ROS was observed in HBEC cells after exposure to CM from NiO NP exposed THP-1*.

In conclusion Ni and NiO NPs induce a release of inflammatory cytokines from exposed macrophages. Furthermore, the detection of secondary genotoxicity in epithelial lung cells by using *in vitro* methods based on conditioned medium or co-cultures was found possible. In order to find out what factors are causing this secondary genotoxicity further investigation is needed.

4.4 STUDY IV: NI AND NIO NANOPARTICLES CAUSE CHANGES LINKED TO EPITHELIAL-MESENCHYMAL TRANSITION (EMT) AND A STEM CELL LIKE PHENOTYPE IN EPITHELIAL LUNG CELLS

BEAS-2B cells were exposed to Ni NPs, NiO NPs and $NiCl_2$ in concentrations of 0.5, 1, 5 and 10 μg Ni/ml for cytotoxicity measurement after 48 h exposure. Significant cytotoxicity was observed in response to Ni at 10 μg Ni/ml as well as for NiO at 5 and 10 μg Ni/ml.

We investigated mRNA expression of eight different genes associated to cancer and EMT after exposure to 1, 5 and 10 μg Ni/ml. Two tumor suppressor genes, *APC* and *CDKN2A* were downregulated by all of the Ni compounds (except no significance for Ni and *APC*). We also found that E-cadherin was downregulated by all of the Ni compounds in the higher doses (5 and 10 μg Ni/ml). No increase of mRNA expression of mesenchymal markers fibronectin, vimentin and N-cadherin could however be observed.

Protein expression of epithelial markers E-cadherin and EpCAM was measured with flow cytometry and a dose dependent down regulation in E-cadherin was observed (significant at the highest doses). A decrease in E-cadherin and an increase in Fibronectin and Vimentin was also confirmed by In-cell western and immunofluorescence (5 μg Ni/ml).

We wanted to investigate if the previous results had implications on a functional level and the scratch wound healing assay was further used (doses 1 and 5 μg Ni/ml) as well as the invasion/migration assay (5 μg Ni/ml). A significant increase in wound healing was observed for Ni 1 μg Ni/ml and $NiCl_2$ 5 μg Ni/ml. The results from the invasion migration showed a significant increase for Ni and NiO NPs.

Further we wanted to investigate if the Ni-exposures could induce a stem cell like phenotype and we investigated expression of three stem cell markers CD24, CD44 and c-KIT. CD24 expression was found to be significantly downregulated both on mRNA and protein level by the higher concentrations in Ni and NiO NPs and $NiCl_2$. c-KIT protein expression was significantly upregulated by the higher concentrations in the exposures as well as mRNA expression (Ni 5 and NiO 10 μg Ni/ml). No upregulation could however be observed in CD44 expression.

In conclusion, both Ni and NiO NPs were found to induce changes linked to EMT and a stem cell line phenotype and the observed effects were comparable for ionic Ni and NPs.

5 DISCUSSION

5.1 PRIMARY GENTOTOXICITY

An important question in the geno-toxicological field is if genotoxicity is caused by direct DNA-interactions or non DNA-reactive mechanisms. In study I we showed that the main mechanism of toxicity was ROS for Ni and NiO NPs. Importantly, none of the Ni-materials induced the ToxTracker reporter related to direct DNA damage and stalled replication forks; a reporter that is activated by many genotoxic carcinogens. In line with these results intracellular ROS was also induced in study II. Other studies have previously demonstrated genotoxicity in the form of DNA-strand breaks in cell models using the same NiO NPs as in this thesis (Kain et al. 2012; Latvala et al. 2016) and others (Ahamed and Alhadlaq 2014; Alarifi et al. 2014; Magaye et al. 2016). An interesting difference between study I and II is that no DNA strand formation and intracellular ROS was formed after exposure to $NiCl_2$ in study I (HBEC, 24 h) whereas this was observed in study II (BEAS-2B, 48 h). There is no clear explanation for these observations other than that the exposure time and cell type differed between the studies. Both cell types are cultured under serum-free conditions, although using different cell media and additives. Furthermore, we showed in study II that Ni and NiO NPs as well as $NiCl_2$ induced chromosomal damage and rearrangements. In this study we also measured the cell uptake (by ICP-MS) and could, importantly, show that Ni and NiO NPs are taken up by BEAS-2B cells while this was not the case for $NiCl_2$. This led to the search for membrane/receptor based mechanisms. Since calcium levels were found to be elevated after exposure to the three Ni-exposures, in line with another study where high doses of soluble green Ni carbonate hydroxide were used (M'Bemba-Meka et al. 2006), we tested the hypothesis that calcium was involved. We found that three different calcium modulators prevented chromosomal damage for Ni and NiO NP exposure and that two of them acted preventive for $NiCl_2$ exposure. In addition the iron chelator deferoxamine was shown to reduce the NiO- and $NiCl_2$-induced increase in MN to levels near the control value. Our results are in line with previous studies which showed a protective effect of both calcium modulators and deferoxamine for the formation of DNA strand breaks and sister-chromatid exchange using soluble green Ni carbonate hydroxide exposed lymphocytes (M'Bemba-Meka et al. 2005; M'Bemba-Meka et al. 2007). Oxidative stress and modulation of intracellular calcium as primary mechanism of genotoxicity may suggest a threshold dose-response compared to agents that interact directly with DNA. This is also supported by the low mutagenic potential observed in study I.

5.2 SECONDARY GENOTOXICITY

In study III we tested the hypothesis that secondary genotoxicity could be induced by Ni and NiO NPs. Using a conditioned media approach as well as a co-culture transwell model we interestingly saw an increase in DNA-strand breaks induced in HBEC cells in both assays. Unfortunately we have not yet found what is causing this effect. In study I we showed lack of DNA strand break induction by $NiCl_2$ in HBEC cells and because of this it is unlikely that the observed secondary genotoxicity would be due to dissolved Ni. One hypothesis is that the

effects are due to ROS produced by macrophages which undergoes oxidative burst. We made various attempts to test increased ROS in the conditioned media using DCFH-DA and another probe HPF but without success so far. Further experiments are needed to find possible factors causing the observed secondary genotoxicity. An assay called Amplex red has previously been used to measure H_2O_2 which could be of interest to us (Wiemann et al. 2016). It could also be of interest to investigate the relationship between cytokines such as IL-β and TNFα and the production of intracellular nitric oxide, as shown before (Green et al. 1994).

To the best of our knowledge, no other studies including NPs and co-culture models investigating secondary genotoxicity using genotoxicity assays as the endpoint have yet been published, hence comparison is difficult. Secondary genotoxicity is noted as the main mechanism of genotoxicity *in vivo*. An issue is that most of the standard *in vitro* models assess primary genotoxicity based on single cell systems which are not always representable for what occurs *in vivo* (Catalan et al. 2017; Evans et al. 2017). Hence, more studies on *in vitro* systems which represent secondary genotoxicity are needed. It is also of importance to develop such systems to attend to the three Rs i.e. replacement, reduction and refinement of animal experiments. The use of a conditioned media approach and a co-culture model was an attempt to better mimic an *in vivo* situation, but in its most simplistic form to study secondary genotoxicity. However, more expensive advanced systems such as 3D cultures and micro tissues could also be used to study the same endpoint.

5.3 NON GENOTOXIC MECHANISMS

In study IV we wanted to study cancer mechanisms not related to genotoxicity. EMT is a mechanism which has been related to metastasis, and loss of E-cadherin is considered to be a hallmark of EMT (Thiery et al. 2009). Interestingly, we observed a clear and dose dependent decrease in E-cadherin at both mRNA and protein level (study IV). Increased protein expression of the mesenchymal markers fibronectin and vimentin was also observed. These results are in line with previous studies (Jose et al. 2018; Tang et al. 2013; Wu et al. 2012) where soluble Ni ($NiCl_2$ and $NiSO_4$) was used, in BEAS-2B cells as well as A549. In addition we found induction of stem cell markers following exposure to $NiCl_2$, Ni and NiO NPs. Our results are further strengthened by functional assays showing increased invasion/migration which was also shown in Jose et al. (2018). In our study the effects of Ni and NiO NPs in addition to $NiCl_2$ was also investigated. To the best of our knowledge there are no previous studies investigating effects on EMT after exposure to Ni and NiO NPs. However a few previous studies showing effects related to EMT as a consequence of NP-exposure have been performed (Chang et al. 2017b; Choo et al. 2016; Gliga et al. 2018; Ma et al. 2017; Song et al. 2017). E-cadherin is a calcium dependent protein and, interestingly, in study II we demonstrate an increase in intracellular calcium after exposure to $NiCl_2$, Ni and NiO NPs. Also a decrease in genotoxicity induced by $NiCl_2$, Ni and NiO NPs was observed after modulating calcium. A study using breast cancer cells showed a transient increase in cytosolic calcium levels after exposure to the stimuli (EGF) used to induce EMT and

induction of EMT markers, including vimentin, was almost completely blocked when chelating intracellular calcium (Davis et al. 2014). One may speculate that E-cadherin decrease could possibly occur because of Ni induced intracellular calcium.

EMT can be induced by inflammation and loss of E-cadherin mediated cell contact has previously been linked to inflammation through induction of NF-κB in cancer cells (Cowell et al. 2009). In study III we aimed to explore if inflammatory cytokines could be released from macrophages following exposure to Ni and NiO NPs. An increase in several cytokines was noted. Interestingly an increase in MIF (migration inhibitory factor), an inflammatory cytokine seen as a pro-tumourigenic factor (Mitchell 2004) was observed. It has been suggested that MIF promotes tumour growth and viability by supporting tumour-associated angiogenesis and modulating immune responses (Chesney et al. 1999; White et al. 2001). A study using cervical cancer cells found that elevation of MIF levels facilitated cell invasion and migration as well as resulted in an increase of vimentin and decrease of E-cadherin expression (Guo et al. 2018). In addition we found an increase of VEGF (vascular endothelial growth factor) which plays a role in pathological angiogenesis associated with tumors (Ferrara et al. 2003) and has been shown to activate NF-κB (Kim et al. 2001; Marumo et al. 1999). Interestingly VEGF mediated angiogenesis was found to link EMT induced cancer stemness to tumour initiation in a study using mouse mammary gland epithelial cells (Fantozzi et al. 2014). VEGF was also found to enhance angiogenic and tumorigenic capacity as well as induce changes leading to gain of EMT characteristics and increased motility of prostate cells (Gonzalez-Moreno et al. 2010). Following 18 h exposure, we noted increased IL-1α and IL-1β, in line with some previous studies (Cao et al. 2016; Gillespie et al. 2010; Morimoto et al. 2010). A study found that IL-1β promotes growth of colon cancer invasion through activation of CSC self-renewal and EMT (Li et al. 2012).

5.4 PARTICLES VS IONS

The nickel ion bioavailability model states that: “the presence of nickel ions in a substance is not sufficient for that substance to be a complete carcinogen” and to act as a carcinogen Ni ions that become bioavailable at the nucleus of epithelial lung cells must be released from the substance. It is suggested that NiO particles are more potent than metallic Ni particles (Goodman et al. 2011). In study II we demonstrated that there were limited to no uptake of $NiCl_2$ while Ni and NiO NPs were taken up by the cells. This observation is in line with the nickel ion bioavailability model as well as other studies (Hack et al. 2007; Harnett et al. 1982) showing that uptake via ion-transport channels of Ni compounds which are soluble in water is not as efficient as uptake via endocytosis of Ni-containing particles for delivering Ni ions to nuclear sites. The results regarding intracellular ROS, DNA-strand breaks and induction of mutations (using the HPRT-mutation assay) using HBEC cells in study I showed that NiO NPs were more potent than Ni NPs whereas soluble $NiCl_2$ showed little (mutations, non-significant) to no potency (ROS and DNA-strand breaks). These results are in line with the theory stated in Goodman et al. (2011) in which NiO harbor higher carcinogenic potential than metallic Ni. Furthermore it is also of importance to consider the fact that Ni and NiO

NPs are expected to induce more severe toxic effects than soluble Ni since their retention in the lungs are higher. This was demonstrated in an *in vivo* study where 40-60% of poorly soluble NiO NPs still remained in the lung after 90 days compared to <0.3% for soluble NiO nanowires (Shinohara et al. 2017).

5.5 IMPLICATIONS FOR ASSESSING RISK

When evaluating risk of a classic toxic compound the limits can be set where no observed adverse effect level can be observed as a threshold derived from a sigmoid curve which shows a dose-effect relationship. However when it comes to compounds which are shown to be genotoxic the principle of setting a threshold is not warranted. The principle that one single molecule is sufficient to irreversibly damage DNA and give rise to tumorigenesis is applied, hence there is no safe dose for genotoxic compounds. Instead, an increase risk is calculated from extrapolating from a dose response seen in animal carcinogenicity experiments (Bos et al. 2004).

We demonstrate genotoxicity of Ni and NiO NPs in study I (comet assay) and study II (comet assay, micronucleus assay) which may raise a concern of genotoxic potential. In study III we also demonstrate evidence of secondary genotoxicity from Ni and NiO NPs. *In vivo* studies are in general given more importance than *in vitro* studies when assessing risk. In a recently developed approach for weighted assessment of the mutagenic potential of nanomaterials, *in vitro* mammalian assays for DNA-damage were ranked with a high uncertainty and low relevance. Furthermore, the comet assay was ranked lower than the micronucleus test and assays measuring unrepairable gene mutations (Catalan et al. 2017). Hence, according to this approach, study II would be ranked higher than study I and they will be implicated in risk assessment with low relevance and high uncertainty.

Lastly in study IV when investigating non genotoxic mechanisms but other mechanisms important in cancer, effects were also observed after exposure to Ni and NiO NPs. Specifically we investigated EMT which is a mechanism not related to genotoxicity but to metastasis. We demonstrate a dose dependent decrease in E-cadherin induced by Ni and NiO NPs, which is a hallmark of EMT. We also demonstrate a decrease of expression in tumor suppressor genes and induction of stem cell markers. However, it is unclear how this data could be used in risk assessment but the future might hold a place for it.

5.6 *IN VITRO* METHODS AND DOSES

In vitro methods, as used in this thesis, are more cost efficient, simpler and faster compared to *in vivo* methods when investigating endpoints of toxicity. The downside using *in vitro* models are that the metabolic activity has not been fully characterized in cell lines and they are not as complex as the human body or animal models. Furthermore there is a problem regarding prediction of biodistribution and target organ toxicity in *in vitro studies* (Kroll et al. 2009). Another important aspect of *in vitro* vs *in vivo* studies is the dosimetry. How do we choose relevant doses for exposure *in vitro* that are relevant and realistic *in vivo*? Some studies use exaggerated doses that are too high to be of relevance in a realistic exposure scenario in

humans. Mathematical models can be used to deal with these concerns (Krug and Wick 2011). Doses of NPs tested in genotoxicity assays are often required to be comparable to possible human exposure scenarios. However human exposure usually occurs at low doses over a long period of time and there may be accumulation of NPs which can make it tricky (Greim and Norppa 2010). To attempt to compare the doses used in this thesis to an exposure scenario the following calculation was used, also used for silver NPs in Wang et al. (2014).

4-week deposition in the lung of a worker exposed to the Swedish hygenic limit of Ni metal total dust: 0.5 mg/m^3. The assumption is that the ventilation rate of a healthy human adult in a working situation is 20 L/min = 9.6 m^3/8 h day). The deposition fraction is 30% and the short term clearance is neglible.

Monthly deposition: 0.5 (mg/m^3) x 0.3 (deposition fraction) x 9.6 (m^3/day) x 5 (days/week) x 4 (weeks) = 28.8 mg/person

Deposition per lung surface area assuming a total surface of 100 m^2: 28.8 /100= 0.288 mg/m^2 surface which is 0.0288 μg/cm^2. To account for uneven deposition: 10 x 0.0288 =0.288 μg/cm^2 ~ 0.29 μg/cm^2.

To put this in comparison to the concentrations used in study I-IV was ranging between 0.21-15.63 μg/cm^2 which puts the calculated value from the example, 0.29 μg/cm^2, in the lower end.

5.7 STRENGTS AND LIMITATIONS OF THE STUDIES

Strengths of the studies used in this thesis include:

- The particles used were well characterized.
- A great number of methods was used to asses mechanisms of carcinogenicity.
- A number of different types of mechanisms of carcinogenicity were studied.
- The lung cell lines was non-cancerous which gives a better chance of mimicking the real exposure of a healthy lung.

Limitations of the studies include:

- Only a single cell line was used in study II, III and IV.
- Only short term exposure was assesed, making it harder to compare to a real exposure scenario.
- The studies were only performed *in vitro* hence their weight in assessing risk will be smaller compared to *in vivo*.

6 CONCLUSIONS

In this thesis different mechanisms of importance for carcinogenicity of Ni NPs, NiO NPs and $NiCl_2$ were studied, as well as similarities and differences in outcome between these exposures. Summary of the conclusions are shown in figure 10. We found:

- Ni and NiO NPs to induce stronger genotoxic effects compared to $NiCl_2$ and identified oxidative stress an important mechanism for genotoxicity rather than direct DNA binding. Mutagenicity was in general shown to be low but a significant increase could be observed for one dose of NiO NPs (study I).

- Ni and NiO NPs as well as $NiCl_2$ to induce chromosomal damage. A mechanism dependent on calcium and iron, but not dependent on cellular uptake was also identified (study II)

- Ni and NiO NPs induce release of inflammatory cytokines from exposed macrophages. Using a conditioned media approach as well as a co-culture model, evidence of secondary genotoxicity was observed, but the factors responsible for the results are still unknown (study III).

- Ni and NiO NPs as well as $NiCl_2$ induce EMT markers, cellular invasion/migration, stem cell markers as well as decrease in mRNA levels of two tumor suppressor genes (study IV).

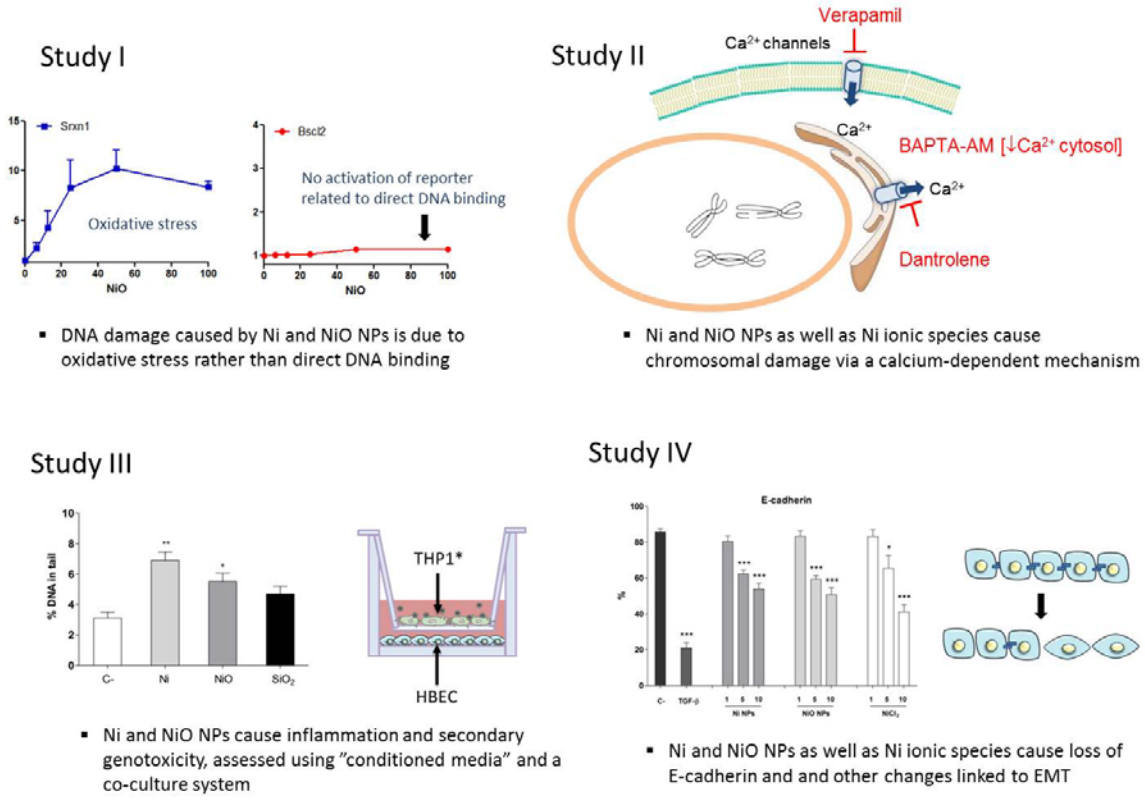

Figure 10. Concluding overview of the studies.

7 FUTURE OUTLOOK

In this thesis a few of the mechanisms mentioned in Hanahan and Weinberg (2011) was investigated i.e. “activating invasion and metastasis” as well as enabling characteristics such as “genome instability and mutation” and “tumour promoting inflammation”. It would be of interest to investigate effects of Ni and NiO NPs on other cancer mechanisms such as “induction of angiogenesis” since we saw an increase of VEGF produced by macrophages in study III. Modulation of calcium’s effect on EMT markers after exposure would also be interesting to study further since we observed effects on genotoxicity in study II.

Furthermore it would be of great interest to expand study III. More efforts needs to be taken to investigate possible factors causing the secondary genotoxicity observed. The study could also be expanded to include more than two cell types. It would be interesting to use primary macrophages and differentiate them to different subtypes and investigate differences in effects after exposure such as differences in secreted inflammatory factors and effects on secondary genotoxicity. I would also be of interest to use more genotoxicity endpoints, than the comet assay, such as the micronucleus assy. The same experimental setup could also be used to study effects of other NPs than Ni and NiO. Furthermore to connect study III to study IV the secreted factors from macrophages effects on EMT in BEAS-2B cells could be investigated.

Secondary genotoxicity is believed to be the main mechanism of genotoxicity noted *in vivo* where cell-cell interplay occurs. Most of the *in vitro* studies in nanotoxicology are performed in 2D and address primary genotoxicity. In study III we used a simple co-culture model to study secondary genotoxicity. In the future there is a need to decrease the amount of animal experiments used in toxicology hence it is of importance to develop more advanced culture systems that better mimic the *in vivo* scenario. More advanced models such as complex 3D-cultures and micro tissues should also be considered.

8 ACKNOWLEDGEMENTS

I would first and foremost like to thank my main supervisor **Hanna Karlsson** for taking me as a PhD student, guidance, positivity, encouragements and scientific guidance. My co-supervisor **Annika Wallberg** for giving me the opportunity to do my PhD and for guidance during my first year. My co-supervisor **Tomas Ekström** for scientific guidance and for letting me perform experiments in your lab.

I would like to thank colleagues in my group **Sebastiano Di Bucchianico**, **Francesca Cappellini** and **Anda Gliga** for collaboration and guidance in the lab. **Sarah McCarrick** for help, support and friendship. **Shafiq Islam** for collaboration, hard work and for giving me the opportunity to supervise.

I would like to thank my collaborators **Inger Odnevall Wallinder, Giel Hendriks, Remco Derr** and **Ernesto Alfaro-Moreno**. Thank you **Sara Skoglund** for helping me with PCCS and nice conversations, **Mikael Ringh** for helping me with pyrosequencing and **Maritha Marcusson Ståhl** for help with multiplex.

Thank you to all the colleagues of Biochemical toxicology and other friends and colleagues from IMM. Thank you **Monika** for friendship, support, taking me to Lithuania, listening to my complaints and lots of help. Thank you **Imran** for friendship, nice conversations, support and company during SOT. Thank you **Katha** for friendship, nice conversations, help and organizing of social events. Thank you **Aram** for guidance, support, friendship and inspiration. Thank you **Kristin** for friendship and nice conversations. Thank you **Rongrong** for friendship, guidance and nice conversations. Thank you **Jessica** for friendship and nice conversations. Thank you to **Mizan**, **Divya** and **Zahra** for friendship, nice conversations and support.

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