Report of the Nickel Ion Bioavailability Workshop

Volume I

February 15-16, 2010
Northern Kentucky University METS Center
Erlanger, Kentucky

Peer Consultation Organized by:
Toxicology Excellence for Risk Assessment
(http://www.tera.org/peer/)

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NOTE

This report was prepared by scientists of Toxicology Excellence for Risk Assessment (*TERA*) and reviewed by the panel members. The members of the panel served as individuals, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

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Executive Summary

A panel of expert scientists met February 15-16, 2010 in Cincinnati, Ohio to discuss a proposed "nickel ion bioavailability" hypothesis for lung tumor induction after inhalation exposure to various nickel substances. The workshop participants conducted a critical evaluation of this hypothesis for lung tumor induction, as outlined in a draft manuscript prepared by Goodman and coworkers (Gradient; Goodman et al., 2010). The goal was to determine whether the hypothesis is biologically plausible and coherent and is supported by the available data.

In particular, the workshop participants discussed the implications of the "nickel ion bioavailability hypothesis" compared to the "nickel ion theory." (The latter is described in the literature as the "nickel ion theory," but is also a hypothesis, and so is described in the rest of this report as the nickel ion hypothesis.) Relevant epidemiological, animal, and *in vitro* data were reviewed and discussed. Workshop participants also identified areas of consensus and areas of disagreement regarding the ability of the different hypotheses to explain the weight of evidence of the available data (epidemiology, animal, *in vitro*), the strengths and weaknesses of existing data to support or refute the hypotheses, the overall weight-of-evidence regarding the respective hypotheses, and the utility of the respective hypotheses in aiding the hazard assessment and/or risk assessment of nickel substances. Suggestions for alternative hypotheses that better support the available data were also considered. Participants were asked to identify data gaps and specific research studies that can be undertaken to validate or disprove the nickel ion bioavailability hypothesis. The intent of the workshop was to focus on the scientific issues and the workshop did not discuss or determine cancer classification for regulatory purposes.

There was unanimous agreement among the workshop panel that the nickel ion bioavailability hypothesis is a refinement and enhancement of the nickel ion hypothesis, and that the nickel ion bioavailability hypothesis represents a transition from earlier ideas, rather than an opposing hypothesis. Thus, the panel concluded that the presentation of the various sections of the Goodman et al. (2010) manuscript (e.g., carcinogenicity, respiratory toxicity, clearance) is generally appropriate, but panel members recommended a number of enhancements and identified additional relevant studies, including several related to the uptake of nickel by the cell. These enhancements and additional data do not affect the overall conclusions of the paper, but reflect some differences in the presentation of specific aspects. The panel agreed that the nickel carcinogenesis is attributed to the nickel ion that is freely available at the target cellular sites[1], and that the ion's bioavailability at the nucleus is critical. While the panel agreed that the form of nickel is important in determining carcinogenesis, it thought that Figure 1 of the manuscript is too simplistic; for example, it does not show any nickel in the nucleus following exposure to soluble nickel. Data supporting differences in uptake of the different forms to the nucleus exist, although these differences are smaller in some experimental systems.

The specific subcellular targets of nickel's carcinogenicity are not known. Nickel delivery to the perinuclear area is a key determinant of carcinogenicity, as addressed by Hack et al. (2007), but it is also important to address the cytoplasmic effects of nickel. The panel noted that the nickel

[1]As used in this report, free nickel ion, or freely available nickel ion, refers to nickel ion that is not bound to proteins; it does not include all soluble nickel.

ion bioavailability hypothesis explains the importance of dose and identifies the dose metric of interest as the amount of free nickel ion that gets to the target – into the cell or the nucleus.

There was considerable discussion about differences and similarities in experimental results for the different forms of nickel, and potential explanations for these observations. The panel distinguished between the nickel ion bioavailability hypothesis, which addresses dosimetry issues, and identification of the mode or mechanism of action for nickel carcinogenesis, considering that the dosimetry implications of the nickel ion bioavailability hypothesis are consistent with a variety of potential modes of action (MOAs). While the mechanism of action for nickel carcinogenesis is not known and there are a number of potential MOAs, the panel reached unanimous agreement that nickel does not act by direct DNA reactivity. Instead, the available data suggest that nickel-induced tumors result from protein-level effects[2]. Various panel members proposed a number of potential MOAs for nickel carcinogenesis, such as oxidative stress, other indirect DNA effects, effects on gene expression and signaling pathways, effects on histone methylation, and protein-induced gene amplification[3]. Immunosuppression was also noted, with particle overload possibly contributing to the observed lung tumor response. The reason for the negative animal studies with nickel sulfate and metallic nickel may have been because toxicity precluded the development of cancer, the cancer potency may have been too low to measure, or these substances may not be carcinogenic in the conventional sense or not reach a practical threshold concentration. The panel recommended that the manuscript note these possibilities, and also address differences in noncancer toxicity among the nickel compounds.

The panel recommended additional areas for enhancement of the manuscript:

- Include results from the Costa laboratory about differences in (1) how the various forms of nickel enter the cell, (2) the rate of uptake, (3) availability (e.g., protein binding and mechanisms and location of release of free ion), and (4) the impact of packaging soluble nickel in liposomes;
- Include results from the Landolph laboratory on uptake of soluble and insoluble nickel compounds and how this correlates with the induction by these compounds of morphological cell transformation (Miura et al, l989; Vekaria et al., 2006); and,
- Note the importance of particle size.

The panel members also agreed that the manuscript should state that the predictions of both hypotheses are consistent with the epidemiology data, but there is somewhat greater consistency with bioavailability being a critical predictor of carcinogenicity. The panel also concluded that, despite the limitations of the epidemiology data, it is clear that some forms of nickel at some concentrations do cause cancer, and that the observed tumors cannot be completely attributed to the effects of confounding exposures. The potential for tumor promotion by soluble nickel was also noted.

[2] Post-meeting, one panel member stated that the tumors result *largely* from protein level effects, noting that nickel ions also cause gene amplification. The mechanism for this gene amplification is not known, as to whether it results from interaction with DNA or with protein.

[3]Post-meeting, one panel member stated that insoluble particles of nickel could have a different MOA from nickel ion, and could enter the nucleus in the particulate form. Another panel member noted post-meeting that in order to enter the nucleus, particles would need to pass through the nuclear pore complex (NCP); i.e., particle size must be smaller than ~40 nm , since the NCP functional diameter is 39 nm (Pante and Kann, 2002).

The nickel ion bioavailability hypothesis was considered a refinement of the nickel ion hypothesis. As early as 1990, IARC noted the importance of generating the nickel ion at critical sites in the target cell. More recent work recognized differences in endocytosis of the different forms, rather than assuming that nickel ion was slowly solubilized from the particulate forms; this concept is now reflected in the nickel ion bioavailability hypothesis. Some data were noted as not supporting the nickel ion bioavailability hypothesis, particularly some individual epidemiology cohorts. However, it was noted that one should look at data as a whole and evaluate trends, rather than focusing on individual cohorts, since, as noted above, there is somewhat greater consistency with bioavailability as a predictor of carcinogenicity. The panel recommended that the manuscript summarize the key aspects of a prior publication by Goodman et al. (2009) regarding the confounding effects of other exposures and the impact of lifestyle.

The panel also recommended that the manuscript provide additional explanation regarding why the nickel ion bioavailability hypothesis was not tested for nasal tumors (because sufficient data to do the analysis are not available), but note that one would expect the same general biology, if data were available. The only difference is that different particle sizes are deposited in the nose and lung. The panel also recommended that the manuscript note that the general concept of the amount of free nickel ion at the target cellular sites being a critical determinant of carcinogenicity would also apply to nasal tumors.

With respect to the issue of thresholds, there was a diversity of opinion. Some panel members do not believe that there is sufficient evidence to prove that a threshold exists for nickel compound carcinogenesis, while others saw support for a practical threshold or similar concept. Panel members suggested a number of issues for the authors to consider, taking into account the ultimate goal of developing an approach for read-across and predicting the carcinogenic potential of less well-studied forms of nickel. Framing the issue in terms of MOA was recommended, since that helps to identify doses and issues of potential concern. While in principle several panel members agreed with the existence of practical thresholds, the panel focused on factors affecting the shape of the dose-response curve and the resulting implications. The panel noted that there are some mechanistic similarities among the different nickel forms, but there are differences on the cancer level, based on the animal bioassays. Comparison with soluble cobalt may also provide insights. Noting that the ultimate mechanism once the nickel ion reaches the nucleus is the same, panel members indicated that the data could be integrated by using differences in dose to the nucleus for soluble nickel vs. subsulfide estimated from the cellular dosimetry model (after suitable checking of the model) to estimate the cancer potency for soluble nickel. (The Hack et al. 2007 paper estimated a 1000-fold difference in nuclear dose.) Another alternative would be to use the approach used by the EU Scientific Committee on Occupational Exposure Limits (SCOEL), deriving an exposure limit on inflammation, based on the idea that protecting against inflammation would also protect against cancer. It was noted that the 1000-fold difference in potency calculated based on dose to the nucleus would not apply to noncancer toxicity endpoints.

Several mechanisms for co-carcinogenesis and/or promotion were proposed, including inhibition of DNA repair, inflammation, oxidative stress, and cell proliferation. Some ideas, including a NFκβ conditional knockout, were brought forward regarding how to test some of the alternatives. A number of other ideas were also raised for data gaps and research needs. Some key research needs identified included measurement of the intracellular nickel concentration following exposure to the various forms, and other data to bridge the gap between the *in vitro* and *in vivo*

data, as well as the evaluation of biological endpoints that are relevant to the carcinogenic process.

1. Participants

Sponsor

Nickel Producers Environmental Research Association (NiPERA)

Authors/Presenters

- Dr. Julie Goodman, Gradient
- Dr. Adriana Oller, NiPERA

Peer Consultation Workshop Panel[4]

- Dr. Ambika Bathija, U.S. Environmental Protection Agency, Office of Water
- Dr. John Bukowski [5], WordsWorld Consulting
  Dr. Harvey Clewell, The Hamner Institutes for Health Sciences
- Dr. Max Costa, New York University School of Medicine
  Dr. Michael Dourson, Toxicology Excellence For Risk Assessment *(TERA),* Chair
  Dr. Andrea Hartwig, Berlin Institute of Technology
- Dr. Uwe Heinrich, Hannover Medical School and Fraunhofer Institute for Toxicology and Experimental Medicine
- Dr. Joseph R. Landolph, Jr., Keck School of Medicine, University of Southern California
- Dr. Len Levy, Cranfield University
- Dr. Günter Oberdörster, University of Rochester
- Mr. Steven K. Seilkop, SKS Consulting Services, Lovelace Respiratory Research Institute
- Dr. Zong-Can Zhou, Peking University Health Science Center[6]

TERA Staff

- Dr. Lynne Haber
- Ms. Melissa Kohrman-Vincent
- Ms. Jacqueline Patterson

[4] Affiliations listed for identification purposes only. Panel members served as individuals at this workshop, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

[5] Dr. Bukowski participated by teleconference.

[6] Dr. Zhou was unable to attend the workshop, but submitted comments that were discussed by the attending panel members.

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2. Background

The peer consultation workshop was organized by Toxicology Excellence for Risk Assessment (*TERA)* with the Nickel Producers Environmental Research Association (NiPERA), which provided funding for the workshop. *TERA* is an independent non-profit organization with a mission to protect public health through the best use of toxicity and exposure information in the development of human health risk assessments. *TERA* has organized and conducted peer review and consultation meetings and workshops for private and public sponsors since 1996 (see www.tera.org/peer for information about the program and reports from meetings). NiPERA, Inc. is a not for profit organization and an independently incorporated division of the Nickel Institute (see www.nipera.org).

The workshop panel was made up of scientists with expertise in the key disciplines necessary to evaluate the proposed approach. The panel members have collective expertise in chemistry, genetic toxicology, nickel and metals toxicology and epidemiology; bioavailability, mode of action for metals; *in vitro* nickel studies; nickel-induced morphological and neoplastic cell transformation and carcinogenesis, cell and molecular biology, risk assessment; and, respiratory toxicity and clearance. Inclusion of knowledgeable experts with a broad range of perspectives is key to the success of the workshop. NiPERA and the Nickel Institute have supported research on nickel toxicity issues by some of the world's leading experts in nickel. Individuals who have been supported by, or have financial ties to, NiPERA, the Nickel Institute (or other nickel interests) were not excluded from this panel, and several of the panel members have received support from NiPERA in the past. These relationships are disclosed in the biographical sketches found in Appendix A. Workshop participants were encouraged to speak their opinions freely and represented their own individual expert opinions, which are not necessarily those of their employers or other groups with whom they are associated or identified. Workshop participants are listed by name and affiliation in this meeting report, but specific opinions and comments are not attributed to individual panel members.

The panel was sent the draft manuscript (Goodman et al., 2010) and a list of discussion questions (found in Appendix A) in early January to ensure adequate time to carefully review the document and prepare for the meeting discussions. Prior to the meeting, panel members provided preliminary comments on issues they thought should be considered. These were shared with the authors and rest of the panel to consider in preparation for the meeting. As these comments were preliminary and panelists may change their opinion upon further review and discussion, they are not part of this final workshop report.

Members of the public were invited to observe the panel discussions by attending the workshop in person. They were also given the opportunity to provide brief oral and written technical comments on the assessment document for the panel's consideration. No written public comments were received, and no observers attended the workshop.

TERA prepared this meeting report. The report summarizes the authors' presentations, the panel discussions, and the authors' comments during the discussions. The meeting report is a summary, not a transcript. Opinions and recommendations of the panel members are noted, although panelists are not identified by name. Panel members have reviewed the draft report,

and their comments and corrections have been incorporated into this final version. The authors also were given the opportunity to review the draft report to confirm the accuracy of their presentations and remarks. This report is available on the Internet at http://www.tera.org/Peer/NiBioavailability/.

3. Panel Introductions, Conflict of Interest, and Meeting Process

The meeting opened with a welcome by Ms. Patterson of *TERA*. She described the background and purpose of the workshop and the agenda for the meeting. Ms. Patterson noted that copies of panel members' biographical sketches and conflict of interest (COI) and bias disclosure statements were available in the attendee folder (see Appendix A). The panel members then introduced themselves and noted whether they had additions or changes in their disclosure statements. None of the panel members had any substantive changes to their statements.

Dr. Dourson, the panel chair, then described how the workshop would be conducted. He explained that discussions would be organized around the discussion questions and would follow the order in the agenda (see Appendix A). He noted that all panelists would have the opportunity to state their own positions on the discussion items and panel members are encouraged to question one another to make sure that all the panel members and the authors understand the scientific basis for the panelist's opinion.

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4. Introduction

4.1 Author Presentations

Prior to the panel beginning its discussions, Drs. Oller and Goodman provided brief presentations. Dr. Oller stated that one motivation for the evaluation in the manuscript was the need to address the health effects of various nickel forms under the European REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) initiative, and to determine the appropriate approach for "read-across" from "reference" nickel substances (i.e., those for which robust animal and *in vitro* data are available) to data-poor chemicals. She and her colleagues are currently using the relative rate of nickel ion release in biological fluids as part of the approach for a qualitative evaluation of carcinogenic potential, and they are interested in guidance on how to use bioavailability information to strengthen the model, as well as what key data sets are needed in order to make the model more useful for read-across. In response to panel questions, Dr. Oller noted that read-across considerations for noncancer endpoints of toxicity are also being considered.

Dr. Goodman introduced the nickel ion bioavailability hypothesis, suggesting that the carcinogenic potency of nickel compounds is proportional to the amount of nickel reaching the nucleus, and contrasting that with the nickel ion hypothesis, which suggests that if the nickel ion can be released from a nickel-containing substance, then that substance should be considered carcinogenic. Dr. Goodman provided an overview of the relevant animal and epidemiology data. She also presented a calculation of the rat tumor Lowest Observed Adverse Effect Concentration (LOAEC), adjusted to a human equivalent concentration (HEC) relevant to the occupational exposure conditions, compared with the estimated exposures for one cohort (Clydach calcining department). A similar analysis was done for all of the epidemiological data, with the idea that, if the nickel ion bioavailability hypothesis is correct, increased cancer risk would be expected when human exposures were higher than the LOAEC(HEC), when expressed in the correct dose units, since exceedence of the LOAEC(HEC) would mean that exposures were in the range where cancers were observed in the animal studies. In response to panelist pre-meeting questions, Dr. Oller explained that the range of HECs corresponding to the rat tumor LOAEC reflects the variability in the particle size distribution (PSD) in different measurements where workers were exposed to that form of nickel. In addition, two approaches were used for calculating the HECs, as shown in Appendix Table A2 of the manuscript, based on either equivalent deposited dose, or equivalent retained dose (correcting for the duration of exposure). A copy of Dr. Goodman's presentation slides is found in Appendix B.

4.2 Supplemental Presentations

Mid-way through the workshop, several participants made additional short presentations of their nickel research in order to provide additional perspective and data relevant to the discussions. A copy of these slides is provided in Appendix B and the presentations are briefly summarized below.

Dr. Hartwig presented data on the effects of nickel on gene mutation and DNA repair (Schwerdtle and Hartwig, 2006). She showed an increase in DNA mutants and DNA adducts

resulting from UV and BPDE (benzo(a)pyrene 7,8-diol-9,10-epoxide) respectively, when there is co-treatment with nickel compounds. She attributes this effect to the inhibition of DNA repair, and noted that it occurs with exposure to both nickel oxide and nickel chloride.

Dr. Oller presented calculations based on the data of Dr. Hartwig, on the levels of soluble nickel that would be required to reach the same intranuclear levels of nickel ion as seen with nickel oxide. Assuming that all of the nickel oxide is bioavailable, she estimated the concentration of nickel in the nucleus following nickel oxide and nickel chloride exposures that result in the same degree of repair inhibition. Based on that calculation, she estimated that *in vitro*, and without clearance, the exposure to nickel from nickel chloride needs to be about 7-fold higher than that from nickel oxide in order to obtain the same concentration of nickel ion in the nucleus. Comparing the nickel lung burden and the inhalation exposure (as mg Ni/m^3) in the NTP (1996a, 1996b) studies, Dr. Oller further concluded that the nickel lung burden from exposure to nickel sulfate at the maximum tolerated dose (MTD) was more than 550-fold lower than the lung burden for nickel oxide at the lowest exposure level at which tumors were found for nickel oxide in rats. Combining these two calculations, she concluded that in order to get the same lung burden and same intracellular concentration as in the nickel oxide study, the exposure to soluble nickel would need to be 7-fold x 550-fold, or 3850-fold higher that the MTD actually tested in the NTP study. Dr. Oller noted that the high intracellular concentration resulting from the uptake of individual particles would mean a larger difference in potency between nickel oxide and nickel sulfate than she had calculated.

Several panel members offered suggestions for these calculations. One panel member suggested that the amount in the nucleus should be compared to the local concentration, not the amount in the petri dish, suggesting that the local concentration may be higher than in the petri dish. The author replied that the assumption of all nickel oxide in the petri dish being responsible for the observed effects is a conservative one, and assumes that the distribution in the petri dish is uniform; it is not known whether the local distribution would be higher or lower. If the bioavailability of Ni from the nickel oxide would be lower, the difference between the compounds would be greater than 7-fold. Several panel members suggested potential approaches to evaluate the exposures of interest to test the calculation. Proposed ideas included using radioactive ^{63}Ni, methods to image small numbers of cells, or lung lavage, although the latter approach would result in multiple cell types. Another panelist suggested conducting an animal study with mixed exposures to soluble and insoluble forms, to mimic occupational exposure conditions.

Dr. Costa presented data on effects of nickel compounds on DNA and uptake into the cell. He noted that nickel subsulfide causes fractionation of heterochromatin in the long arm of the X chromosome, but this effect is not seen with soluble nickel. He noted that packaging nickel chloride in liposomes increases the uptake significantly over nickel chloride in solution, and this increase is further enhanced by bovine serum albumin (BSA); once packaged, the nickel chloride can cause heterochromatin fractionation. He further noted that the uptake of a single particle of nickel subsulfide results in a high intracellular nickel concentration. Dr. Costa presented fluorescence data showing that nickel subsulfide is taken up by cells much faster than nickel chloride, and that nickel subsulfide reaches the nucleus relatively rapidly. He also noted a recent publication in Biochemistry (Ellen et al., 2009) that presents a conceptual model in which nickel ion replaces the magnesium ion in binding to the phosphate backbone of DNA. The nickel ion condenses chromatin better than magnesium, ultimately resulting in the silencing of tumor

suppressor genes. Dr. Costa also noted that nickel subsulfide causes much more oxidative stress in the nucleus than does soluble nickel.

Dr. Clewell presented on the nickel cellular dosimetry model he published with Hack et al. (2007). He noted that different forms of nickel are cleared primarily by different mechanisms, with nickel oxide being primarily extracellular, nickel subsulfide primarily intracellular, and the soluble forms (nickel sulfate and nickel chloride) being transcellular[7]. The Hack model describes the differences in the cellular uptake and intracellular kinetics of the different classes of nickel compounds. The model helped identify key data gaps. The ultimate goal would be a model incorporating deposition and clearance. Hack, Clewell and colleagues applied the model to predict the amount delivered to the lung cells *in vitro*. Dr. Clewell noted that issues described in the context of Dr. Oller's presentation regarding calculating the particle concentration in the vicinity of the cell would also apply to the Hack et al. (2007) model, and he noted that Teeguarden et al. (2007) addressed the issue of "particokinetics" *in vitro,* calculating particle concentration in the vicinity of cells. However, the model predicted that the concentration of the nickel ion in the nucleus would be 1000-fold higher following exposure to nickel subsulfide *in vitro* than following exposure to nickel chloride. This difference could explain the difference in tumor response in the experimental animal bioassays. Dr. Clewell noted that validation of the model is needed, and recommended that data be collected using lung microdissection or imaging following inhalation exposures.

A panel member noted that kinetic data in alveolar epithelial cells are needed, since the test system used for the *in vitro* analyses were A549 cells, a human epithelial cell line derived from lung carcinoma tissue. Dr. Clewell noted that it is assumed that the target for the lung tumors would be a tracheobronchial cell in humans, while the alveolar region is often a target in rodents. He also stated that the estimated 1000-fold difference in intracellular dose accounted for differences in cellular clearance, and clearance from lung, but did not consider redistribution. With large caveats, the model predicts a 1000-fold difference in potency between nickel subsulfide and soluble forms. Panelists suggested that the Goodman et al. manuscript include information on the Hack et al. (2007) model, and that it show the predictions of the cellular dosimetry model.

Dr. Landolph presented data on cell transformation induced by various forms of nickel and various nickel samples. He noted that green nickel oxide, black nickel oxide, nickel subsulfide, and crystalline nickel monosulfide, which are all insoluble nickel compounds, all strongly induce morphological transformation of C3H/10T1/2 mouse embryo fibroblasts in culture. He further stated that soluble nickel sulfate does induce morphological cell transformation, but the potency for this compound is far lower than for the insoluble nickel compounds. The potency of nickel sulfate in inducing morphological transformation of C3H/10T1/2 cells is either 0 (Miura et al., l989) or very small (Vekaria et al., 2006). Studies on the induction of morphological and neoplastic transformation of C3H/10T1/2 mouse embryo cells were conducted with two nickel refinery dust samples. One sample was collected from a nickel refinery prior to the 1920s, and contained 10% arsenic in the form of orcelite (Ni_5As_2). The second sample was from 1929, and contained only 1% arsenic, after processes were changed and the arsenic was largely removed

[7] Post-meeting, Dr. Clewell clarified that intracellular clearance refers to endocytosis by the alveolar epithelial cells resulting in high cellular concentrations. Transcellular clearance refers to transport through the cells into the systemic circulation, without producing high concentrations in the cells.

from the sulfuric acid used in the nickel refining process (Clemens and Landolph, 2005). There were similar levels of endocytosis with both forms, but only the pre-1920s sample produced cell transformation, and it did so in a dose-dependent manner, consistent with the hypothesis that the observed tumor response in the workers is due to the orcelite, alone or in combination with the green nickel oxide also present in the 1919 sample. The presenter believes that this work shows that the ability of these two samples to induce morphological cell transformation correlates with the potential for the refinery dust at different times to cause nasal and respiratory tumors in humans working there. It was suggested that this sort of transformation system could be used for read-across for REACH. One of the authors agreed that this would be a useful model system, but wondered whether a cell transformation system in human cells would be more valuable. However, a panel member noted that that there are no routinely used, reliable, reproducible assays that are standardized and widely accepted among scientists to detect chemically-induced transformation of human cells.

4.3 Clarifying Questions and Workshop Framing

The panel asked several clarifying questions that led into a discussion of the framing of the workshop and manuscript.

The panel questioned what the term "bioavailable" means in this context. One panel member noted that soluble nickel is rapidly cleared, and small amounts enter the cell, so that bioavailability means not just the amount that enters the tissue, but the amount that is taken up by the cell. Panel members also noted that the nickel ion needs to become bioavailable *in* the nucleus, not at the nucleus. Several panelists defined bioavailable as freshly dissolved nickel ions in the cell, also known as "free" nickel ions (since the ions rapidly bind to cellular constituents). Free nickel ion was defined as consisting of loosely, rapidly-reversibly bound ion and unbound ion. (Henceforth, as used in this report, free nickel ion, or freely available nickel ion, refers to nickel ion that is not bound to proteins; it does not include all soluble nickel.) One panel member noted that when cells are treated with nickel subsulfide and nickel chloride, the subsulfide is more strongly reactive with chromatin. Both forms do get into the nucleus, but the soluble form enters more slowly.

In considering the overall purpose of the workshop and manuscript, several panelists commented on the approach of the manuscript of contrasting the nickel ion bioavailability model with the "nickel ion theory." Several members suggested that, as stated, the "nickel ion theory" is too simplistic; the expectation/belief that soluble nickel is a more potent carcinogen than nickel subsulfide has fallen out of favor. One panelist noted that historically several leading nickel researchers did believe in the nickel ion hypothesis (described in the literature as the "nickel ion theory"), but the current consensus is that differences in uptake to the cell are important. Another panelist agreed, noting that there has been an evolution and a transition in thinking about what determines nickel carcinogenicity, and bioavailability clearly predicts carcinogenicity better than solubility does. **Overall, there was unanimous agreement among panel members that the nickel ion bioavailability hypothesis is a refinement and enhancement of the nickel ion hypothesis, and that the nickel ion bioavailability hypothesis represents a refinement of earlier ideas, rather than an opposing hypothesis.** However, a practical need for the work of the panel was noted. Given that some scientists have endorsed the nickel ion hypothesis in a risk assessment, and then a regulatory context, it may be important to note the dichotomy between the two approaches.

Panel members made several suggestions for clarifying Figure 1 of the paper (reproduced below without modification). The authors explained that the intent was to read the figure and accompanying text from the top down, showing how each successive step affects the final result of how much nickel ion gets into the nucleus, rather than comparing across forms for a given step. A panel member suggested that, rather than showing relative toxicity, that the actual maximal tolerated dose (MTD) be presented for the different forms. Another stated that water-soluble nickel is taken up by the nucleus, since effects on DNA repair are observed in *in vitro* studies. Showing a small arrow entering the nucleus would convey the gradation of ease and amount of nickel reaching the nucleus. A panelist also noted the need to account for the longer retention time of insoluble particles *in vivo*.

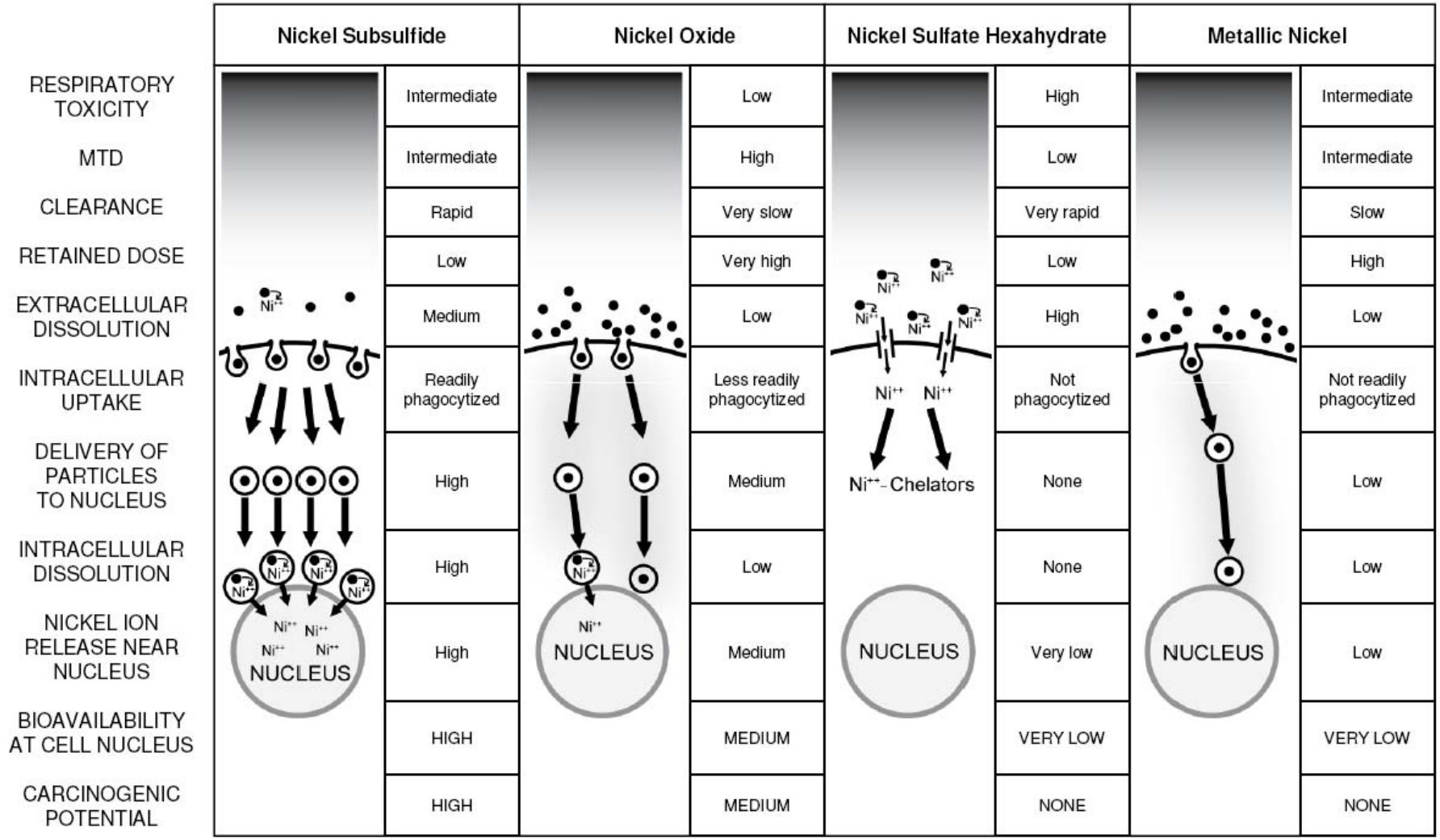

Figure 1. The nickel ion bioavailability model takes into account the various factors that determine the bioavailability of the nickel ion at the nucleus of target cells and the carcinogenic potential of nickel-containing substances (from the Goodman et al. (2010) report).

5. Panel Discussion

The panel discussed the discussion questions that were distributed to panel members with the draft manuscript.

5.1 Questions 1 and 4

The panel discussed Questions 1 and 4:

> ***Question 1: In vitro and Experimental Animal Data***
> ***Do the available in vitro and in vivo data support the conclusions of Goodman et al. (2010) regarding: a. carcinogenicity of the various forms in animals b. respiratory toxicity c. clearance d. cellular uptake (ion transport, phagocytosis) and intracellular dissolution e. transport to the nucleus? Are there other available data (either supportive or contrary) relevant to the above and is there potential for alternative interpretations of the data regarding nickel carcinogenicity in animals?***
>
> ***Question 4: Are there other hypotheses that might explain the data better than the bioavailability model (e.g., a tumor-promoting mechanism that does not depend on direct nuclear interactions; or evocation of tumors based on lung inflammation, the nickel ion hypothesis, the amount of nickel inhaled or retained in the lung, or something else)?***

In addressing the *in vitro* and experimental animal data, the panel considered a number of aspects, including the carcinogenicity of the various forms in animals, *in vivo* clearance, cellular and nuclear uptake, the impact of particle size and noncancer toxicity, and possible mechanistic explanations for the carcinogenic effects of nickel compounds.

One of the panel members noted the low incidence of lung tumors in the nickel sulfate study (NTP, 1996a), and asked whether one would expect a statistically significant increase if the number of animals per group were larger. Another panelist replied that one could do a power analysis, asking whether the result would be statistically significant if there more animals per group and the percent response were the same. The panelist noted, however, that this analysis would not be meaningful, since one cannot assume that the response rate would be exactly the same as that observed in the NTP (1996a) study. Another panelist stated that it is inappropriate to ask if the same response rate would have been statistically significant with a larger sample size; if the study was negative, it was negative.

In addressing the differences among the forms, one panel member suggested that the tumor outcome from nickel oxide exposure may have been partially due to the particle effect, rather than just the nickel ion. Another panelist noted that he had conducted calculations related to the particle load, and concluded that even the high concentration of nickel oxide did not result in sufficient accumulation to result in particle overload. The first panelist suggested that the tumors may have resulted from the combination of the effects of nickel ion and particle loading. A panel member recommended that the panel consider the impact of particle size, noting that inhalation of copper nanoparticles causes oxidative stress, but soluble copper compounds do not. The difference is related to different uptake mechanisms, and the potential for nanoparticles to

result in high intracellular nickel concentrations. Nanoparticles of a certain size may be able to enter the nucleus via the nuclear pore complex.

A panel member noted that, consistent with the nickel ion bioavailability hypothesis, nanoparticle exposure can result in a high internal concentration of nickel in the cell when dissolution occurs intracellularly, especially at acidic pH (as is present in the lysosome). In response to a question about particle sizes under occupational conditions, one of the authors stated that different instrumentation than that used for the available exposure analyses would be needed to capture nanoparticles, and she is not aware of any measurements conducted in nickel refineries in that size range. The panelist suggested that there is the potential for generation of nanoparticles in refining operations, since such particles are formed whenever metals are heated to high temperatures.

In integrating and evaluating the data, a panelist suggested that one first should think in terms of dosimetry, and then in terms of mode of action (MOA)/mechanism. Both the nickel ion bioavailability hypothesis and the nickel ion hypothesis indicate that the nickel ion is the ultimate carcinogenic agent, and then the question becomes one of identification of the target – the lung surface, the cell, or the nucleus. Unlike many chemicals, for which blood levels are a good surrogate for tissue levels, the work of Costa and colleagues and that of Landolph and colleagues indicates that for nickel, the concentration in the blood or at the lung surface is not a good surrogate. Instead, one needs information on the intracellular concentration. Determination of whether the effects of nickel are a result of interactions in the nucleus or in the cell cytoplasm is a separate, mechanistic question.

The panelist further noted that, while it is difficult to conduct *in vivo* studies that measure intracellular concentrations, the Hack et al. (2007) model used *in vivo* and *in vitro* data to predict intracellular and intranuclear concentrations. A key conclusion was that the concentration in blood is not a good surrogate for the nickel ion concentration in epithelial cells. *In vivo* clearance alone cannot be used to estimate the nickel ion concentration in lung airways. Validation of the model would require doing micro-dissection of airways (as has been done by Buckpitt and Plopper) and measuring the nickel ion concentration in airway epithelial cells. While tissue to do these studies may have been archived from the NTP studies, it was noted that this technique is tedious. The Hack et al. (2007) paper also recommended studies that are needed to validate and improve the cellular dosimetry. The ultimate goal of the Hack et al. team was to connect the cellular dosimetry model to a pharmacokinetic model.

One of the authors noted that the nickel ion bioavailability hypothesis refers to the amount of nickel reaching the nucleus, not systemic bioavailability. She noted that the working hypothesis needs to explain the differences observed between the different forms of nickel, and why nickel sulfate was negative for carcinogenicity in both inhalation and oral studies. If the target is the cell membrane (rather than the nucleus), one would expect a positive tumor response in the oral study based on the high blood concentration after oral exposure, but soluble nickel was negative following oral exposure.

In considering the overall database, a panelist recommended that additional information be provided on how different forms of nickel enter cells, since this information helps to integrate the *in vitro* and *in vivo* observations. This panelist noted that soluble nickel enters the cell as ions that rapidly bind to proteins, so that little free nickel ion is available. When cell cultures are

exposed to nickel subsulfide and nickel sulfate, damage to heterochromatin is seen only with nickel subsulfide, but not with nickel sulfate. However, if the soluble nickel is packaged in liposomes, protecting the ion from interacting with protein, damage to heterochromatin does occur. By contrast, nickel subsulfide enters the cell in endosomes, an acidic environment that leads to release of free nickel ions. The endosomes migrate to near the nucleus, releasing reactive, free nickel ions from nickel subsulfide near the nucleus. This panelist suggested that these data (citations included in the References section) should be cited in the manuscript. Others agreed that endocytic uptake of insoluble nickel is important, noting that the degree of uptake correlates well with cell transformation efficiency. A panelist noted that, consistent with the results of Hack et al. (2007), delivery of nickel ion to the perinuclear area is a critical determinant of carcinogenicity, but dose to the cytoplasm also needs to be considered. Panel members considered the observations reflecting differences among nickel forms in cellular uptake mechanisms to be consistent with the conclusion that the difference among the forms is primarily due to differences in dose delivered to the nucleus. They suggested that the dose of interest is freshly dissolved nickel ion (free ion), because nickel ion rapidly binds protein, and nickel that is bound to protein is less reactive.

The panel discussed the available data regarding the potential for different forms of nickel to enter the nucleus and interact with nuclear constituents, and the implications for nickel carcinogenicity and MOA. One panelist noted that studies conducted with a dye that binds only nickel ions provide data on the amount of nickel ion that entered living cells after exposure to nickel subsulfide and nickel chloride. Such studies are available *in vitro*, but not from *in vivo* exposures. A potential enhancement to this work would be to include a wash-off study to evaluate kinetics. Another panelist noted studies that measured nickel in the nucleus following *in vitro* exposures to nickel chloride or nickel oxide (see references by Hartwig and colleagues in the References section). Both forms of nickel inhibited the repair of DNA adducts and oxidative DNA damage. Specifically, in an *in vitro* study with equitoxic doses of nickel oxide and nickel chloride, twice as much nickel ion entered the nucleus in the former case. A panelist noted that studies of nickel binding in the nucleus require breaking open the cell, which can affect the binding measurements. DNA repair has not been measured *in vivo* for these different nickel forms. However, Kasprzak et al. (1997) found that oxidative DNA damage *in vivo* was observed after a 48-hour exposure period, but not after 24 hours, implying the accumulation of DNA damage, which can be interpreted as repair inhibition. The observation of the inhibition of repair implies that soluble nickel reaches the nucleus under the test conditions. This panelist hypothesized that the high toxicity of nickel sulfate and metallic nickel may be the reason that no carcinogenicity was seen for these forms in the rodent studies.

Another panelist noted the distinction between differences related to dosimetry and those related to carcinogenic potential. The latter issue asks whether there is a substance (e.g., nickel ion) that causes cancer at the target, regardless of how it reaches the target. Once the active agent is identified, one can focus on what forms of nickel allow the nickel ion to enter the cell and approach the nucleus. This panelist stated that the question is whether nickel needs to penetrate the nucleus in order to exert its effects. Another panelist noted that nickel subsulfide is constantly dissolving, and the newly released ion is very reactive; another panel member preferred considering the newly released ion as "reactive". The first panelist suggested that the appropriate dose metric may be the amount of newly dissolved nickel ion. The panelist also noted that nickel subsulfide is more tightly bound in the nucleus than soluble nickel, suggesting that the difference may be because of the higher concentration of nickel ions in the nucleus from

the nickel subsulfide particles compared to following exposure to soluble nickel, or because soluble nickel was already bound to protein when it entered the nucleus.

A panelist raised the question of what causes the pulmonary toxicity of the various forms of nickel, and why that is higher in the rat than in humans. Another panelist suggested that the toxicity results from nickel interacting with proteins in the cell.

Noting that nickel is also immunotoxic, one of the authors suggested that the observed oxidative damage could be secondary to inflammation. One of the panelists noted that inhaled nickel chloride is a model compound for oxidative stress, but oral exposure does not result in lung inflammation.

The panel then turned to a more detailed discussion of potential mechanisms and MOAs. **While neither the mechanism nor MOA of nickel carcinogenesis is known, there was unanimous agreement by the panel that nickel does not act by direct DNA reactivity.** Instead, the available data suggest that the tumors result from protein-level effects.[8]

Although not the focus of the workshop, panel members proposed a number of potential MOAs/mechanisms of carcinogenic action for nickel compounds. It was noted that these mechanistic ideas are generally a refinement of the main hypothesis, reflecting what happens once the nickel ion gets into the cell. The following potential MOAs/mechanisms of action were proposed:

- Particle overload was suggested as an enhancer, based on the internal doses for nickel oxide, although doses were not high enough for this to be a primary cause of the tumors.
- Oxidative DNA damage/oxidative stress; this could be related to particle load.
- Immunosuppression compromising the body's response to initiated tumors.
- A variety of indirect DNA effects. For example, nickel can displace essential metals (e.g., zinc) from DNA repair proteins, inhibiting the function of DNA repair enzymes. Indirect mutation was proposed, based on observed chromosomal instability and accumulation of DNA damage with extended *in vitro* exposure. Binding to the phosphate backbone in place of magnesium was also noted.
- Epigenetic effects on gene expression, resulting from the inhibition of oxidative iron-dependent histone demethylases.

The panel discussed some of these potential MOAs in more detail. One panelist noted that 24-hour treatment with nickel subsulfide causes gene silencing, due to methylation of histone H3K4, but this effect is not seen with nickel chloride unless treatment is prolonged (for 3 weeks). The panelist attributed the difference to nickel subsulfide particles sticking to the membrane, so that the true treatment period after washout was longer than 24 hours. This sort of issue and the impact of *in vivo* clearance need to be considered in evaluating the results with the different forms of nickel.

The panel discussed, but did not fully resolve, the cellular localization of the target(s). One panel member suggested that the bioavailability may refer to presence in both the cell and in the

[8] Post-meeting, one panel member stated that the tumors result *largely* from protein level effects, noting that nickel ions also cause gene amplification. The mechanism for this gene amplification is not known, as to whether it results from interaction with DNA or with protein.

nucleus, rather than solely the nucleus. Several mechanistic hypotheses for the carcinogenic effect of nickel were suggested. One member noted that there are many effects of nickel on signaling pathways that can occur without nickel entering the cell. This raised the question of whether the amount in the nucleus is the correct dose metric. Nickel can activate jnk and NFκβ without entering the cell, and can stabilize and activate the hypoxia inducible factor 1 α (HIF-1 α) transcription factor, making the cell react as if it is hypoxic. The implications of these effects are not known, nor is it known whether such gene activation from extracellular effects occurs in vivo. Inhibition of oxidative iron-dependent histone demethylases can also result in changes in gene expression via epigenetic mechanisms. Another panel member stated that critical targets may be proteins, rather than DNA or other nuclear targets. A third noted that many exposures affect the same pathways but do not cause cancer; the question is what nickel does to cause cancer. Another panelist noted that nickel may also cause carcinogenesis by the formation of reactive oxygen species (ROS), and selective activation of stress-signaling pathways induced by nickel and its compounds may play an important role in carcinogenesis. Several panelists recommended that the manuscript authors address the broad spectrum of nickel effects, including respiratory toxicity and inflammation, since these effects can cause cancer via indirect DNA effects, although it was also noted that inflammation was observed in rats with all forms of nickel. Panelists suggested that the authors could focus on the nucleus, but they do need to address these other effects in more detail. Inclusion of additional information on cell signaling and cell proliferation was recommended.

The panel discussed whether any of the proposed mechanisms help differentiate the results expected among the different forms. One panel member noted that there are large differences in the amount of oxidative stress caused by the different forms, with more oxidative stress from particulate forms than soluble forms.

In considering mechanistic explanations for the animal and epidemiology findings, one panelist noted that exposures other than nickel (e.g., irritation from acid mists) could have accounted for some of the risk observed in some of the epidemiology studies. Another panelist stated that the risk cannot be totally "explained away" as due to confounding; specifically, the occurrence of (normally rare) nasal cancers in workers in different nickel refinery operations (e.g., electrolytic, pyrometallurgical) suggests a causal role for nickel, as opposed to confounding exposures. A third panel member noted a study he has conducted illustrating the potential for confounding by other exposures. He tested a refinery sample from 1919, and a sample obtained from the same refinery in 1929 (after a process change was made), and found that the early (1919) sample caused morphological cell transformation, with a dose-response curve that appeared to be composed of transformation by orcelite and green nickel oxide added together, but the later (1929) sample did not induce cell transformation. The early (l919) sample contains 10% arsenic, in the form of nickel arsenide, or orcelite, while the 1929 sample only contains 1% arsenic in the form of orcelite, suggesting that the orcelite (containing arsenide) together with the green nickel oxide, may have been responsible for the cell transformation (Clemens et al, 2003).

One of the authors noted that a key purpose of the manuscript under consideration is to determine what differences among the different forms lead to the observed differences in tumor response in the rat bioassay. She noted that inflammation alone cannot be the explanation, since inflammation occurs both under conditions where tumors are observed and in the absence of tumors. Among the various concentrations and nickel forms tested in the bioassays (NTP, 1996a; 1996b; 1996c), there was only one test group with no inflammation, the low

concentration of nickel sulfate; inflammation was observed with nickel sulfate at the higher concentrations, but there was no increase in the tumor response.

One panel member noted the potential for epigenetic effects, resulting from the inhibition of oxidative iron-dependent histone demethylases, which can result in the activation or repression of gene expression. This panel member noted that epigenetics is the reason that tissues have different phenotypes.

In summary, there was unanimous agreement that the nickel ion bioavailability hypothesis is an extension and enhancement of the nickel ion hypothesis, and that it represents a refinement of earlier ideas, rather than an opposing hypothesis. Thus, the panel concluded that the presentation of the various sections of the Goodman et al. (2010) manuscript (e.g., carcinogenicity, respiratory toxicity, clearance, etc.) is generally appropriate, but they recommended a number of enhancements and identified additional relevant studies, including several related to the uptake of nickel to the cell. These enhancements and additional data do not affect the overall conclusions of the paper, but reflect some differences in the presentation of specific aspects. The panel agreed that the nickel ion is the toxic moiety, and that carcinogenesis is attributed to the freely available nickel ion, and that its bioavailability at the nucleus is critical. The form of nickel is important in determining carcinogenesis, but Figure 1 of the manuscript is too simplistic, for example, not showing nickel at all in the nucleus following exposure to soluble nickel. There are data supporting differences in uptake of the different forms to the nucleus, but these differences are smaller in some experimental systems. The specific subcellular targets of nickel are not known. Nickel delivery to the perinuclear area is a key determinant of carcinogenicity, as addressed by Hack et al. (2007), but it is also important to address the cytoplasmic aspect. The panel noted that the nickel ion bioavailability hypothesis explains the importance of dose and identifies the dose metric of interest as the amount of free nickel ion that gets to the target – into the cell or the nucleus.

There was considerable discussion about differences and similarities in experimental results for the different forms of nickel, and potential explanations for these observations. The panel distinguished between the nickel ion bioavailability hypothesis, which addresses dosimetry issues, and identification of the potential MOA or mechanism of action for nickel carcinogenesis, considering that the dosimetry implications of the nickel ion bioavailability hypothesis are consistent with a variety of MOAs. While the mechanism of action for nickel carcinogenesis is not known and there are a number of potential MOAs, the panel reached unanimous agreement that nickel does not act by direct DNA reactivity. Instead, it was suggested that the tumors result from protein-level effects (with one panel member considering tumors to result *largely* from protein-level effects). Various panel members proposed a number of potential MOAs for nickel carcinogenesis and recommended that the manuscript note these possibilities. Several of these were epigenetic effects, including oxidative stress, other indirect DNA effects, effects on gene expression and signaling pathways, effects on histone methylation, and protein-induced gene amplification. Immunosuppression was also noted, with particle overload possibly contributing to the observed tumor response. The reason for the negative animal studies with nickel sulfate and metallic nickel may have been because toxicity precluded the development of cancer, the cancer potency may have been too low to measure, or that these substances may not be carcinogenic in the conventional sense or may not reach a practical threshold concentration. Therefore, it is also important to address differences in noncancer toxicity among the nickel compounds.

The panel recommended additional areas for enhancement of the manuscript:

- Include results from the Costa laboratory about differences in (1) how the various forms of nickel enter the cell, (2) the rate of uptake, (3) availability (e.g., protein binding and mechanisms and location of release of free ion), and (4) the impact of packaging soluble nickel in liposomes
- Include results from the Landolph laboratory on uptake of soluble and insoluble nickel compounds and how this correlates with the induction by these compounds of morphological cell transformation (Miura et al, l989; Vekaria et al., 2006)
- Note the importance of particle size.

5.2 Question 2

> ***Question 2: Epidemiology Evidence - Goodman et al. (2010) conclude that the epidemiological data support both the nickel ion hypothesis and the bioavailability hypothesis. They conclude that the epidemiological data are not sufficiently robust for determining which hypothesis is most appropriate, but are consistent with the nickel ion bioavailability hypothesis. Do the available epidemiology data support this conclusion? Could the data support a different conclusion? Do the data support one hypothesis over another? Should other available data be discussed?***

One panelist began the discussion by summarizing the epidemiology data with respect to the hypotheses and recommended that a number of issues be noted in the manuscript. It was this panelist's opinion that the available epidemiological data can support either the nickel ion hypothesis or the nickel ion bioavailability hypothesis. Because the exposure levels of the different nickel species are moderately to strongly correlated in almost all of the cohorts, it is hard to tease out which form(s) play a causal role in carcinogenesis. The situation is further complicated because there are no exposure measurements for the early 50-60 years of exposure, when exposure was highest. Each nickel species is both an exposure and also a confounder relative to the others. Estimates have been made for these early exposures, but that is not as good as actual measurements. The potential for misclassification of the form of nickel, along with the former issues, makes the distinction in carcinogenic potency among various nickel forms based on the epidemiology data an intractable problem.

The panelist stated that the Goodman et al. (2009) review article did a better job than the draft Goodman et al. (2010) manuscript of addressing confounding by other species and other exposures, and the impact of lifestyle, and recommended that these issues be addressed in more detail in the current manuscript. Potentially confounding exposures include high dust levels, acid mists, combustion products, and smoking. Making the analysis more difficult, nickel exposures were highest at the time that the other (potentially confounding) exposures were also highest. This panelist stated that it is clear that some level of some nickel species or combination of species is carcinogenic, but the epidemiological data do not clearly point to which form or level. The panelist noted that the confounding exposures include a number of irritants, which could affect the overall quantitative tumor response, in addition to any carcinogenesis from these other exposures.

Drawing an analogy to cigarette smoke, the panelist noted that the overall exposure was carcinogenic, but the data are not sufficient to tease out which components are responsible for which portion of the response. The panelist also recommended that the manuscript address how "data mining" and issues of multiple comparative analyses might affect the results of the epidemiological literature, noting that it is quite common for epidemiological researchers to perform numerous exploratory analyses looking for positive results, and then to preferentially report those that best fit the investigators' preconceived hypotheses. For example, Grimsrud and colleagues (Grimsrud et al., 2002) analyzed the impact of insoluble forms of nickel adjusting for soluble nickel, but not the converse (soluble nickel adjusted for insoluble forms). Another panel member noted that these data were the basis for IARC considering soluble nickel as carcinogenic. This panel member believes that a hypothesis that is consistent with all of the epidemiological data, as articulated by the ICNCM (1990), is that soluble nickel exposure enhances the tumor response associated with co-exposure to some insoluble nickel forms, and stated that the manuscript should note the mixed exposures and the potential for tumor promotion. The first panel member also noted that there are some epidemiology studies showing an almost perfectly monotonic dose-response for soluble nickel, suggesting that the cutpoints and model construction were designed to maximize the degree to which water-soluble nickel was implicated in the cancer response. Another issue with the epidemiology data is that studies that conducted modeling often did not show model diagnostics. The panelist stated that a reality check of the results is needed. For example, the Easton et al. (1992) paper found that removing metallic nickel as an explanatory variable had a large effect on the model coefficients without changing the goodness-of-fit (GOF). The absence of effect on GOF suggests that there also should not have been an effect on the model coefficients; the observed changes could have been related to the co-linearity of exposures.

Another panelist suggested that the manuscript be revised to state that the epidemiology data support the bioavailability hypothesis more than the nickel ion hypothesis, rather than stating that the epidemiology data equally support both hypotheses. This panelist recommended that evaluation of Table 4 of the manuscript should look for trends, rather than at absolutes. A panelist noted that the version of Table 4 in the authors' presentation (Slide 11, Appendix B**),** is useful to help the reader see trends, and noted that the risk at Clydach went down dramatically among workers who started after 1940, a change that correlates with lower sulfidic nickel exposure and with lower arsenic exposure and lower exposure to nickel arsenide (orcelite; see Landolph's laboratory results, Clemens and Landolph, 2003). Several panel members suggested doing a sensitivity analysis, using different cutoffs for grouping the data, and seeing if the cutoffs affect the interpretation of the results. Noting the uncertainty in the actual exposure measurements, one panel member suggested using a 2-way plot of data showing the confidence in both exposure and risk as intervals, and offered to provide an example. [NOTE: the panelist provided an example post meeting, and it is found in Appendix C].

Considering the implications of the epidemiology data for the respective hypotheses, several panel members noted that the nickel ion hypothesis was an early hypothesis, and the bioavailability hypothesis is a more refined version of the nickel ion hypothesis, recognizing that nickel kinetics need to be taken into account, but that the nickel ion is the carcinogenic form. Events occurring in the cell are important, not just systemic bioavailability.

The panel also concluded that, despite the limitations of the epidemiology data, it is clear that some forms of nickel at some concentrations do cause cancer, and that the observed tumors

cannot be attributed to the effects of confounding exposures. The potential for tumor promotion by soluble nickel was also noted.

The panel made a number of recommendations for enhancements to the manuscript.

- Note issues with the epidemiology data, as discussed by Goodman et al. (2009):
  - Co-occurrence of exposures to the various nickel forms;
  - potentially confounding exposures, including smoking, other forms of nickel, acid mists;
  - potential for selectively cutting data until get a good fit is obtained;
  - absence of information on model diagnostics in studies with models;
  - analyses done in one direction, not other (e.g., controlling for soluble nickel in insoluble nickel analysis, not vice versa); and,
  - need to reality check results.
- Include a 2-way plot of data showing confidence in both exposure and risk.
- Note potential for tumor promotion via soluble nickel toxicity.

5.3 Question 3

The panel next discussed the overall support for the proposed hypothesis (Questions 3-8).

> ***Question 3. How strong is the overall integration of the in vitro data, and human and experimental animal data (by relevant routes of exposure) to support the bioavailability hypothesis. What evidence is counter to this proposed hypothesis?***

In considering the overall integration of the data and alternative hypotheses, the panel focused on data that could be interpreted as being inconsistent with the hypotheses (focusing primarily on the bioavailability hypothesis).

Reflecting on how the epidemiology data relate to the two hypotheses, one of the panel members said that two cohorts could be seen (and are often viewed by regulators) as arguing against the bioavailability hypothesis, based on substantially elevated lung cancer risks where soluble nickel exposures dominated. The first cohort is the electrolytic workers at Kristiansand, although there were marked differences in the exposure estimates for this cohort between ICNCM (1990) and Grimsrud et al. (2003). The second cohort is nickel refinery workers at Outokumpu Oy, Finland. Although Table 4 of the manuscript shows both of these cohorts as having exposure to sulfidic nickel above the LOAEL(HEC), this is based on uncertain estimated sulfidic nickel exposure ranges, which contain values that generally did not exceed the LOAEL(HEC). In the case of the Kristiansand refinery workers, in only one of seven work areas within electrolysis operations was ICNCM's estimated average sulfidic nickel concentration in excess of the LOAEL(HEC). For the Finnish refinery, the upper bound of the estimated sulfidic exposure range shown in Table 3 of the manuscript (0.02-0.4 mg Ni/m^3) barely exceeded the most conservatively estimated LOAEL(HEC) of 0.36 mg Ni/m^3 shown in Table A2 of the manuscript. Another panel member suggested that the refining workers at Huntington Alloys (before 1947) may also be inconsistent with the bioavailability hypothesis, since levels were above the rat tumor LOAEL(HEC), but there was no increase in lung tumor risk. The first panelist suggested that the absence of evidence of increased risk may have been due to dilution, rather than being a true negative (most of the workforce was not engaged in calcining, with high levels of nickel subsulfide exposure).

One panel member asked whether most of the people in the epidemiology cohorts who got cancer were smokers, but another responded that the lung cancer risks were still substantially increased over those expected in occupational cohorts with higher smoking rates than the general populations against which they were compared.

In considering how the animal data relate to the two hypotheses, the panel framed the discussion in terms of the mechanistic and mode of action studies. With regard to the bioavailability hypothesis and alternative hypotheses, one panel member stated that the difference between soluble and particulate forms of nickel reflects a gradation of effect, rather than a yes/no situation. The panel member noted that effects of nickel ion have been observed in the nucleus following *in vitro* exposure to soluble forms of nickel (Schwerdtle and Hartwig, 2006). The Schwerdtle and Hartwig (2006) study was done at equitoxic concentrations of nickel oxide and nickel chloride, and compared such endpoints as the inhibition of DNA repair by these two forms. Another panelist noted issues with comparing exposures to particulates and soluble nickel, noting that it is hard to characterize the concentration of particulates in an *in vitro* medium, due to the issue of gravitational settling; the concentration of interest is really the amount of particulate in contact with the cells.

One of the authors added additional information related to the comparison of soluble and particulate forms, referring to her presentation (found in Appendix B) showing that, in order to reach the same concentration in the nucleus of human lung cells in an *in vitro* study, exposure to nickel chloride needs to be about 7-fold higher than the exposure to nickel oxide (when expressed in terms of nickel concentration). A panelist stated that particulate forms are more toxic than soluble forms for the same molar concentration, based on the LD_{50} in *in vitro* studies (Fletcher et al., 1994). This panelist stated that the comparisons described by the author expressed molar concentrations as if the particles dissolve completely in the medium, but the particles (1-5 microns) are being deposited on top of cells. Another panelist noted the difficulty of comparing concentrations, due to the issue of particle deposition on cells.

In summary, the panel noted that the nickel ion bioavailability hypothesis was a refinement of the nickel ion hypothesis. As early as 1990, IARC noted the importance of generating the nickel ion at critical sites in the target cell. More recent work recognized differences in endocytosis of the different forms, rather than assuming that nickel ion was slowly solubilized outside the cell from the particulate forms; this idea is now reflected in the bioavailability hypothesis. Some data were noted as not supporting the bioavailability hypothesis, particularly some individual epidemiology cohorts, but it was noted that one should look at data as a whole and evaluate trends, rather than focusing on individual cohorts. It was also recommended that the manuscript summarize the key aspects of the Goodman et al. (2009) paper regarding the confounding effects of other exposures and the impact of lifestyle.

5.4 Question 6

> ***Question 6. The bioavailability hypothesis focused on lung cancer. ICNCM (1990) also found that several forms of nickel were associated with increased nasal cancer risk in the epidemiology studies, but nasal cancer was not reported in any of the experimental animal studies with inhaled nickel. Should the bioavailablity hypothesis (or other hypotheses addressing nickel carcinogenicity) consider other tumor types in addition to the lung?***

A panelist noted that, although the analysis in the manuscript was restricted to lung cancer, there are suggestions from the epidemiology data that the nose may also be a potential target organ for cancer, and recommended that this should also be addressed in the manuscript. One of the authors responded that, in the NTP (1996a, 1996b, 1996c) studies, no nasal tumors were observed, even though 50% of the deposition occurs in the nose of rats. In addition, there are no data on clearance from the nose. Another panelist noted differences in the anatomy of the human and rat nose, particularly the presence of sinuses in humans but not rats, and the faster clearance from the rat nose. With regard to how the mechanistic hypotheses would relate to nasal tumors, one panel member suggested that one would expect epidemiologically-based trends similar to those observed for lung cancer, as there was a strong correlation between the cohorts with elevated lung tumors and those with elevations in nasal tumors. However, nasal tumors are rare and there were much smaller numbers of nasal cases than lung cancer cases in the exposed cohorts, making it more difficult to see a significant signal with respect to risks associated with exposure to different nickel forms.

Based on these comments, panel members suggested that the manuscript should provide additional explanation regarding why the bioavailability hypothesis was not tested for nasal tumors (because sufficient data to do the analysis are not available), but note that one would expect the same general biology, if data were available. The only difference is that different particle sizes are deposited in the nose and lung. The panel also recommended that the manuscript note that in the development of nasal tumors the general concept of the amount of free nickel ion at the target being a critical determinant of carcinogenicity would also apply.

5.5 Question 5

> ***Question 5. In focusing on nickel reaching the nucleus, the authors suggest that, even if the effects of the nickel ion in the nucleus are assumed to be via genotoxicity, a "practical threshold" for initiation of carcinogenicity exists. Please comment on this assertion.***

A panel member began the discussion by commenting that, in principal, he agrees that practical thresholds exist. However, he noted that describing a practical threshold depends on an understanding of the critical event(s) for carcinogenicity. If the carcinogenic potential depends solely on the concentration of the nickel ion in the nucleus, does the delivery system (e.g., soluble vs. insoluble nickel) matter? This panelist noted that the Scientific Committee on Occupational Exposure Limits (SCOEL) recommended that the occupational exposure limit (OEL) for all nickel compounds be based on inflammatory changes to the lung, as a crude approach. This approach assumes that the inflammatory changes are a key trigger for cancer, but uncertainties in that assumption were noted. Another panel member noted that most of the proposed mechanisms for nickel carcinogenicity reflect either indirect genotoxicity or are epigenetic. A third panelist stated that the mechanism for morphological and neoplastic cell transformation induced by insoluble nickel compounds (green nickel, black nickel oxide, and crystalline nickel monosulfide, and nickel subsulfide) is not completely known, but appears to involve nickel-induced gene amplification (of the Ect-2 gene), and gene silencing. Approximately 130 genes are differentially expressed in nickel transformed cells, including increased expression of oncogenes (Ect-2 gene) and decreased expression of tumor suppressor genes (Landolph et al, 2002; Verma et al, 2004). This panelist stated that it is not known

whether there is a connection between chromosome breakage and cell transformation. This panel member noted that the cell transformation curves are linear with dose, and extrapolate through zero morphological cell transformation (response) at zero concentration/dose of insoluble nickel compounds. This panelist believes that one molecule of nickel ion can trigger genetic instability, if it interacts with the appropriate target (proto-oncogene or tumor suppressor gene or protein causing silencing of tumor suppressor gene). Hence, this panelist indicated that there are no strong data indicating a threshold for nickel compound-induced morphological or neoplastic cell transformation or carcinogenesis, and he does not at present believe such a threshold exists. Another noted that the *in vitro* studies with nickel compounds were not conducted at very low doses, and suggested that if there is a threshold, it could be at a very low dose. Panelists drew an analogy to arsenic, which has a low threshold (less than 0.1 μM) for cellular effects with the effects of interest resulting from protein interactions. However, it is not known whether the threshold for arsenic is relevant to the approach for quantification of human cancer risk. If the threshold is lower than arsenic exposures from food and water, human exposure would be above the threshold, and a linear extrapolation would still be relevant, despite the existence of a threshold.

One panel member asked how the hypothesized low threshold relates to data suggesting a pronounced threshold for nickel oxide for lung tumors in rats. Another responded that this observation reflects the differences between a biological threshold and a mathematical threshold, with the animal data showing an apparent threshold due to lower sensitivity, although the dose-response would be nonlinear[9]. It was also noted that the risk assessment community is shifting focus from the fundamental biology of the shape of the curve at low doses, to considering the impact of human variability relative to the concentration at which effects are seen. This panelist agreed with the suggestion by one of the authors that the nickel sulfate data suggest the presence of a practical threshold in animals.

Another panel member suggested that the primary effects of nickel result from interaction with protein, but noted that the MOA is not known, and there is no clear sequence of key events. The SCOEL assessment addressed the impact of high toxicity, noting that if workers are protected from toxic effects, they would likely also be protected from carcinogenic effects of nickel.

One panel member stated that all agree that soluble nickel is not carcinogenic by inhalation in animals. This panelist further noted that regulatory bodies dealing with carcinogens require strong evidence supporting the presence of a threshold in order for that to be accepted in a regulatory context, and that such data for nickel carcinogenesis do not exist at present. Another stated that a practical threshold is suggested by the observation that nickel is at most weakly genotoxic, and that soluble nickel is inefficient at penetrating the cell and reaching the nucleus. Others replied that the issue is identifying the dose of the practical threshold, and noted the

[9] The panelist further explained this comment post-meeting as follows: The situation I was describing is sometimes called "lurking genotoxicity". It arises when there is equivocal evidence regarding genotoxicity, but tumors are only observed at concentrations associated with cytotoxicity and increased cell proliferation. Clearly, one would expect a dose-dependent transition at the dose where cytotoxicity and cell proliferation occur, but it is not possible to distinguish between the two possible alternatives: (1) the mode of action is strictly epigenetic (e.g., chloroform), and genotoxicity does not play any role in the tumorigenesis, so the tumor risk below the dose-dependent transition would be expected to be zero, or (2) genotoxicity does play a role in the tumorigenicity, but above the dose-dependent transition the potency is greatly increased by the effects of cytotoxicity and cell proliferation, so the failure to observe tumors below the dose-dependent transition reflects inadequate dose group size, not the absence of tumorigenicity.

importance of bringing an understanding of MOA to the evaluation of the data and assessment of human relevance. Noting that the ultimate mechanism once the nickel ion reaches the nucleus is the same, it was suggested that the data could be integrated by using the factor of 1000 difference in dose to the nucleus for soluble nickel versus nickel subsulfide to estimate the cancer potency for soluble nickel. This would capture the shades of grey in the carcinogenic potential for soluble nickel, but would mean that the non-cancer "safe" concentration would drive the exposure limits for this form of nickel.

Noting that one of the aims of this workshop was to develop an approach for addressing forms of nickel for which data are lacking, two of the panel members, noted that the approach used by the SCOEL provides a useful solution. That approach used inflammation as the basis for limiting exposure, based on the idea that protecting against inflammation would also protect against cancer. This approach does not use all of the MOA data, but is useful based on the available data. A panel member also noted that individual cells may have high internal concentrations of nickel ion after uptake of a single particle, so it may not be appropriate to compare the average concentration of soluble and particulate nickel in the lung. Another noted that toxicity probably occurs due to cytoplasmic interactions, so the 1000-fold difference in potency calculated based on dose to the nucleus would not apply to noncancer endpoints.

One panel member noted that there are differences in the cellular targets activated by soluble and insoluble forms, and the forms differ in nuclear uptake, although there is some overlap in target (e.g., HIF-1 α). Another drew an analogy to benzo[a]pyrene (BaP) diol epoxide, which interacts covalently with DNA and is a good mutagen and tumor initiator, but needs to be generated inside the cell in order to exert its mutagenic and carcinogenic effects. Similarly, the issue for soluble nickel is how much gets into the cell. Another panel member noted that soluble nickel competes with the divalent metal transporter-1 (DMT-1), depleting iron in the cell, but subsulfide enters the cell via endocytosis, and so does not affect iron levels. This panelist noted that there are some (*in vitro*) endpoints where nickel subsulfide and soluble nickel have similar effects and potencies, and some where there are qualitative differences. This panelist suggested that soluble nickel and soluble cobalt be compared mechanistically, since soluble cobalt is very carcinogenic; the differences between the metals may be illuminating for nickel. The panelist further suggested that the difference may be due to the potential for Fenton chemistry with different ionization states of cobalt, versus the absence of Fenton chemistry with nickel.

Based on the panel discussion, one of the authors suggested conducting an experiment in which rats are exposed to cobalt sulfate, nickel sulfate, and nickel subsulfide, at exposure levels that cause similar toxicity and at which cobalt sulfate and nickel subsulfide have caused tumors, but not nickel sulfate. Then one could look for markers of toxicity (e.g., cell proliferation, apoptosis) and cancer MOA and see if different patterns appear that may explain the differences in tumor outcome and suggest what MOA is relevant for each of these compounds. Panel members agreed, noting that one could do immunohistochemical staining of HIF-1 α or downstream target genes such as CA-9 or NDG1. It was also noted that the methylation of Histone H3K4 increases if nickel reaches the nucleus. The need for data in exposed people was also noted. The question was raised whether cytotoxicity *in vitro* is the same as *in vivo* cytotoxicity; a panel member suggested looking at individual cells (e.g., with immunohistochemical staining), rather than homogenized tissue, to address these questions.

One panel member noted that it is important to address potency differences between insoluble forms, as well as differences in delivery systems. Another noted that a paper by Sunderman et al. (1987) compared multiple endpoints for the various forms of nickel.

In summary, there was a diversity of opinion with respect to practical thresholds. Some panel members do not believe that there is sufficient evidence to prove that a threshold exists for nickel compound carcinogenesis, while others saw support for a practical threshold or similar concept. The panel members suggested a number of issues for the authors to consider in the context of thresholds, taking into account the ultimate goal of developing an approach for read-across and predicting the carcinogenic potential of less well-studied forms of nickel. Framing the issue in terms of MOA was recommended, since that helps to identify doses and issues of potential concern. While in principle some panel members agreed with the existence of practical thresholds, the panel focused on factors affecting the shape of the dose-response curve and the resulting implications. It was noted that there are some mechanistic similarities among the different nickel forms, but there are differences on the cancer level, based on the animal bioassays. Comparison with soluble cobalt may also provide insights. Noting that the ultimate mechanism once the nickel ion reaches the nucleus is the same, the data could be integrated by using the 1000-fold difference in dose to the nucleus for soluble nickel versus subsulfide estimated from the cellular dosimetry model (suitably checked) to estimate the cancer potency for soluble nickel. Another alternative would be to derive an exposure limit based on inflammation, following the idea that protecting against inflammation would also protect against cancer. It was noted that the 1000-fold difference in potency calculated based on dose to the nucleus would not apply to noncancer endpoints.

5.6 Question 8

> ***Question 8. Are there other issues or questions that should be discussed relative to the nickel ion bioavailability hypothesis and its relevance to understanding the potential for carcinogenicity from nickel exposure?***

The panel discussed the data regarding the potential for soluble nickel to be a promoter. One panel member noted that in epidemiological studies with similar levels of soluble nickel exposure, increased risk is associated with more co-exposure to insoluble nickel, suggesting some interaction between the different forms. The panelist also noted that cell proliferation is seen *in vivo* with nickel subsulfide, but not with nickel sulfate. Another panelist noted that soluble nickel causes oxidative stress in the lung, and *in vitro* studies find apoptosis, cell death, regenerative cell proliferation with soluble nickel. One panel member noted that the data do not allow the distinction between soluble nickel acting as a tumor promoter (which acts after an initiator) and as a co-carcinogen (which is administered at the same time as the initiator). This panelist noted that promotion has specific mechanistic implications. This panelist noted that a key question is whether soluble nickel can promote the effects of insoluble nickel compounds, and that this can really only be tested well in animal carcinogenicity studies. The panelist recommended that this experiment should be done to determine whether or not soluble nickel has tumor promoting effects in animals Another panelist suggested that inhibition of DNA repair (a co-carcinogenic mechanism) could explain the epidemiological observations.

It was noted that several chemicals (e.g., chloroform, formaldehyde) cause tumors by causing cell death followed by regenerative cell proliferation, but it is not known why some cytotoxic

chemicals such as chlorine do not cause tumors. One panel member suggested that the difference may be that in some cases the proximate carcinogen does not reach the inside of the cell.

In response to a question, one panel member stated that EPA did not explicitly use the data on promotion by soluble nickel, noting that even if it is considered a promoter, it is still a carcinogen. Kasprzak et al. (1990) conducted an initiation/promotion study, with sodium barbital, but the sodium barbital itself caused tumors, making it difficult to interpret the results. A panel member suggested using an NFκβ conditional knockout, in which the protein is not expressed in the lung. NFκβ is a major inflammatory transcription factor that is activated by nickel, arsenic, and other metals; using a knockout would help in determining the role of inflammation in carcinogenesis. A limitation is that the knockout is available only in mice, and nickel-induced lung tumors were observed in rats, but not mice.

In summary, several mechanisms for co-carcinogenesis and/or promotion by soluble nickel were proposed, including inhibition of DNA repair, inflammation, oxidative stress, and cell proliferation. Some ideas, including a NFκβ conditional knockout, were brought forward regarding how to test some of the alternatives.

5.7 Questions 7 and 9

> ***Question 7. Can an overall weight of evidence conclusion be made at this time? If not, what further analyses might help?***
>
> ***Question 9: Data Needs. Data needs are identified and discussed in the manuscript. Should additional data needs be added or deleted? Please rank the data needs according to which are essential to identify the determinants of nickel carcinogenicity and explain the differences observed among the various forms.***

The panel addressed these two discussion questions together, beginning with a discussion of the research needs identified in the draft manuscript. One panel member noted that the cost to address all of the noted questions would be substantial, in the millions of dollars.

One panel member noted that the available *in vitro* data can be integrated with the *in vivo* experimental animal results to yield several credible MOAs, and the MOA information can be used to evaluate the differences in potency of the different forms. The intracellular nickel model of Hack et al. (2007) and PBPK modeling can be used to improve the credibility of the read-across. This panel member identified *in vivo* measurements of intracellular nickel concentration as a critical data need. Several methods for measuring intracellular nickel levels were suggested. Measurements can be made *in vivo* using ^{63}Ni and autoradiography of the lung. This has been done by Agneta Oskarsson with ^{63}Ni-labeled soluble nickel (Oskarsson and Tjälve, 1979; Oskarsson et al., 1979). Similar studies could be done with nickel subsulfide and nickel chloride synthesized from radiolabeled ^{63}Ni. Nose-only exposures could be conducted to reduce the amount of material needed. While one panel member suggested that quantification would be hard on the cellular level, one of the authors noted that Benson and colleagues (2002) did a study to try to co-localize Ni in the cellular organelles of rat lung tissue after whole body inhalation exposure to nickel sulfate or nickel subsulfide. Lung tissue blocks from exposed animals and controls were cut into three thin sections. The two outer sections were histologically stained and

the middle section was analyzed using proton-induced X-ray emission (PIXE). The data set from the control rats showed no presence of Ni anywhere within the scanned area. The Ni image from the sulfate sample showed no significant Ni localization. Due to the low concentration of Ni, the image looks rather like a "random array of dots." The resulting Ni image from the Ni subsulfide sample showed significant Ni localizations, with Ni concentrations in the scans ranging from <1 mg/kg to 1 wt% (four orders of magnitude). It was suspected that the high concentrations could be indicative of particles of Ni. Unfortunately, this technique did not allow the mapping of the particles to any specific extra or intracellular sites. Another panelist suggested that the method used by Hartwig and colleagues (Schwerdtle and Hartwig, 2006) could be applied.

One panel member suggested that the animals be exposed, and then the nuclei isolated and evaluated to determine if nickel reached the nucleus. It would also be useful to evaluate which signaling pathways (e.g., NFκβ) are activated by nickel exposure. It would be useful to evaluate how entry to the nucleus varies with the particle size, and how the size of nanoparticles affects their interaction with DNA. Transmission electron microscopy (TEM) could be used to visualize whether particles are getting into the nucleus. Evaluation of multiple time points after exposure, and co-exposures of ionic and particulate forms of nickel would help the assessment. It would also be useful to evaluate how soluble forms of nickel interact with the epithelial lining fluid and bind to proteins. Another panelist also flagged interest in co-carcinogenic effects, and suggested conducting a cell transformation assay or other intermediate assay with a mixture of soluble and insoluble forms of nickel.

Since the draft manuscript noted questions with respect to nickel-copper oxide, one panelist asked whether additional studies on this compound would be useful. Another panelist noted that this exposure is of interest in light of the potential for occupational exposure to nickel-copper oxide. Information about the effects of inhalation exposure to copper alone would help in interpreting the data. The authors noted that the issue of the nickel-copper oxide was raised both in trying to understand some of the epidemiology data, and in trying to determine whether the nickel-copper oxide behaves more like nickel subsulfide or nickel oxide. One panel member suggested that a cell transformation study be done before *in vivo* studies. One of the authors noted that Sunderman published on the bioavailability of different nickel-copper oxides. A panelist noted that metallothionein chelates copper well.

Another panelist recommended that *in vivo* mechanistic data be collected, and that information on repair inhibition and nuclear uptake be collected. This panelist also suggested that more information on metallic nickel is needed, including *in vitro* data. Another stated that he has published on cell transformation, uptake, and cytotoxicity of metallic nickel (Costa et al., 1981). The need to consider the implications of nanoparticles was raised, but one of the authors noted that all forms of nickel would need re-testing to consider the implications of exposure to nano-size materials. A panelist stated that the metallic nickel used in the inhalation bioassay (Oller et al., 2008) is different from the powder used in the *in vitro* studies by Costa and colleagues, and it would be useful to have *in vitro* data on the same material as used in the bioassay.

In response to a panelist question, one of the authors noted that metallic nickel develops a thin film of the oxide, but because the film is only one atom thick, the metallic nickel particles behave differently from nickel oxide. One panel member suggested that metallic nickel might initially interact with the cell as the oxide, and then behave differently later, but one of the authors noted that there are differences in how metallic nickel and nickel oxide enter the cell.

Another panel member noted that the metal is less potent than the oxide at transforming cells, and suggested that a fluorescent dye could be used to see if nickel ions are released from the metal particles.

Noting that none of the forms of nickel cause cancer in mice, a panel member suggested that it would be fruitful to explore the interspecies differences between rats and mice, and investigate how strongly the toxicity of the nickel ion is related to carcinogenicity. Gene expression studies in mice and rats could help explain the mechanism of carcinogenicity and why tumors are not observed in mice. This panel member also recommended that more information be obtained from human lung tissue, and comparisons be conducted of the effects in mouse, rat and human tissue slices. The panelist noted that, using precision-cut slices, tissues can be kept vital for a week, and exposures can be conducted using methods similar to cell culture. Cellular and nuclear uptake, and markers of toxicity can be measured. This panelist also noted that Pott et al. (1987) found that intratracheal instillation of nickel metal powder resulted in a high incidence of tumors. These tumors have been attributed to particle overload rather than a specific effect of nickel, but it is not clear why no effect was seen with nickel oxide, which does cause inflammation. The panelist noted that other studies by Pott et al. (1989, 1992) tested the effects of i.p. injection of stainless steel particles containing different percentages of nickel, and found that the higher percentages of nickel correlated with higher tumor response. Another panelist agreed that any insoluble particulate can cause overload at high enough exposures, and that overload can increase the effect of the particle, even when the effect is not due solely to overload.

5.9 Final Comments

Some panelists took the opportunity to make additional concluding comments.

One panelist stated that the weight of evidence of the animal and epidemiology data provides a clear picture for the insoluble forms, and the evidence is fairly strong that there is no evidence of metallic nickel carcinogenicity, but the data are equivocal regarding soluble nickel. This uncertainty points to the need for mechanistic data, because epidemiology data will not be able to provide a clear answer. Another noted the need for collecting *in vivo* data to validate the *in vitro* data. This panelist stated that a systemic model exists for nickel, including a deposition and clearance model, but data need to be collected on other forms and in the right cells to verify the cellular disposition model.

The need to identify parameters that correlate with carcinogenesis was noted, with possible candidates including morphological and neoplastic cell transformation (Miura et al., l989; Landolph, l994; Landolph et al, 2002), amount of nickel ions in the nucleus (as in the work of Hack et al., 2007), and perhaps binding to histones; more comparisons of *in vitro* and *in vivo* data were recommended. Another panelist recommended more work in animal models, and in human lung tissue if the system behaves like *in vivo* data. The panelist recommended visualization of particles in lung epithelial cells, using cultured Beas-2B immortalized human bronchial epithelial cells. These cells are immortalized, and can be transformed to anchorage independence, although one other panelist pointed out that these cells are tumorigenic.

Another panel member noted that dose- response data are needed for all *in vitro* and *in vivo* studies. Rat type I and type II cells can be used as a test system, although Type I cells are the

predominant cell type (~95 % of the alveolar surface), and one can evaluate the initiating signal for cell proliferation.

A panel member stated that the data show that nickel subsulfide and nickel oxide are carcinogenic, and nickel sulfate could be carcinogenic, based on the mechanistic data. This panelist suggested comparing the potency of the different forms, since the data support a consistent mechanism (related to free nickel ion). The results of the PBPK/cellular uptake model that estimates large internal differences in nuclear dose for the different nickel forms provide a potential explanation of the negative results in the rat bioassay with nickel sulfate; the difference appears to be in potency, rather than a lack of carcinogenicity of soluble nickel. This panel member also recommended additional studies *in vivo* or in human respiratory tissues, and noted the need to protect sensitive populations in the context of environmental exposures. Another panelist noted the need to identify the factors that lead to sensitivity. Another panel member indicated that soluble nickel compounds are not carcinogenic in animals and induce either no (Miura et al., l989) or at most weak (Vekaria et al., 2006) morphological transformation in cell culture.

With regard to sensitive populations, one panel member stated that low-level exposures to soluble nickel would not be expected to cause cancer preferentially in children. Conversely, added sensitivity of the elderly is not expected, since they would die of other causes before the end of the latency period.

Overall, a panelist noted that the current *in vitro* data are not sufficient to determine the mode/mechanism of action for nickel carcinogenicity. Information on delivery to the nuclear target sites (bioavailability) is critical. An important research need is a study to bridge between the *in vitro* and *in vivo* data, looking at biological endpoints that are most relevant to the carcinogenic process.

6. References

Benson, J. M., March, T. H., Hahn, F. F., Seagrave, J.-C., Divine, K. K., Belinsky, S. A. (2002) Final report for short-term inhalation study with nickel compounds. Lovelace Respiratory Research Institute. Albuquerque, NM.

Clemens, F., and Landolph, J. R. (2003) Genotoxicty of samples of nickel refinery dust. Toxicological Sciences, 73: 114-123.

Clemens, F., Verma, R., Ramnath, J., and Landolph, J. R. (2005) Amplification of the Ect-2 proto-oncogene and over-expression of Ect2 mRNA and protein in nickel compound and methylcholanthrene-transformed 10T1/2 mouse fibroblast cell lines. Toxicology and Applied Pharmacology, 206: 138-149.

Costa, M., Simmons-Hansen, J., Bedrossian, C.W., Bonura, J., Caprioli, R.M. (1981) Phagocytosis, cellular distribution, and carcinogenic activity of particulate nickel compounds in tissue culture, Cancer Res 41(7):2868-2876.

Easton, D.F., Peto, J., Morgan, L.G., et al. (1992) Respiratory cancer mortality in Welsh nickel refiners: Which nickel compounds are responsible? In *Nickel and Human Health: Perspectives* (Eds.: Nieboer, E., Nriagu, J.O.). John Wiley 7 Sons, Inc. p. 603-619.

Ellen, T.P., Kluz, T., Harder, M.E., Xiong, J., Costa, M. (2009) Heterochromatinization as a potential mechanism of nickel-induced carcinogenesis. Biochemistry, 48(21):4626-4632.

Fletcher, G., Rosetto, F., Turnbull, J., et al. (1994) Toxicity, uptake, and mutagenicity of particulate and soluble nickel compounds. Environ Health Perspect, 102:69-79.

Goodman, J.E., Prueitt, R.L., Dodge, D.G., Thakali, S. (2009) Carcinogenicity assessment of water-soluble nickel compounds. Crit Rev Toxicol, 39(5): 365-417.

Goodman, J.E., et al. (2010) The nickel ion bioavailability model of the carcinogenic potential of nickel-containing substances. Prepared for Nickel Producers Environmental research Association (NiPERA).

Grimsrud, T.K., Berge, S.R., Haldorsen, T., Andersen, A. (2002). Exposure to different forms of nickel and risk of lung cancer. Am J Epidemiol, 156(12): 1123-1132.

Grimsrud, T.K., Berge, S.R., Martinsen, J.I., Andersen, A. (2003). Lung cancer incidence among Norwegian nickel-refinery workers 1953-2000. J Environ Monit, 5: 190-197.

Hack, C.E., Covington, T.R., Lawrence, G., et al. (2007). A pharmacokinetic model of the intracellular dosimetry of inhaled nickel. J Toxicol Environ Health Part A ,70:445-464.

ICNCM (International Committee on Nickel Carcinogenesis in Man). (1990) Report of the international committee on nickel carcinogenesis in man. Scand J Work Environ Health,

16(1): 1-82. Report to US EPA; Commission on European Communities; Energy, Mines, and Resources Canada; National Health and Welfare Canada; Ontario Ministry of Labor; Nickel Producers Environmental Research Association.

Kasprzak, P., Diwan, B. A., Konishi, N., Misra, M., Rice, J. M. (1990) Initiation by nickel acetate and promotion by sodium barbital of renal cortical epithelial tumors in male F344 rats. Carcinogenesis, 11(4):647-652.

Kasprzak, P., Jaruga, P., Zastawny, T. H., et al. (1997) Oxidative DNA base damage and its repair in kidneys and livers of nickel(II)-treated male F344 rats. Carcinogenesis, 18: 271-277.

Landolph, J. R. (1994) Molecular mechanisms of transformation of C3H/0T/2 Cl 8 mouse embryo cells and diploid human fibroblasts by carcinogenic metal compounds. Envir. Health Perspect. 102(Suppl. 3): 119-125.

Landolph, J. R., Verma, A., Ramnath, J., and Clemens, F. (2002) Molecular biology of deregulated gene expression in transformed C3H/10T1/2 mouse embryo cell lines induced by specific insoluble carcinogenic nickel compounds. Environ. Health Perspect., 110(suppl 5): 845-850.

Miura, T., Patierno, S. R., Sakuramoto, T., and Landolph, J. R. (1989) Morphological and neoplastic transformation of C3H/10T12/ Cl 8 Mouse embryo cells by insoluble carcinogenic níckel compounds. Environmental and Molecular Mutagenesis, 14: 65-78.

NTP (National Toxicology Program). (1996a) NTP report on the toxicology and carcinogenesis studies of nickel sulfate hexahydrate (CAS No. 10101-97-0) in F344/N rats and B6C3F1 mice (Inhalation Studies). National Institute of Environmental Health Sciences, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC; NTP TR 454; PB 96-3370.

NTP (National Toxicology Program). (1996b) National Toxicology Program. NTP report on the toxicology and carcinogenesis studies of nickel oxide (CAS No. 1313-99-1) in F344/N rats and B6C3F1 mice (Inhalation Studies). National Institute of Environmental Health Sciences, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC; NTP TR 451; PB 96-3367.

NTP (National Toxicology Program). (1996c) National Toxicology Program. Toxicology and carcinogenesis studies of nickel subsulfide (CAS No. 12035-72-2) in F344/N rats and B6C3F1 mice (Inhalation Studies). National Institute of Environmental Health Sciences, Public Health Service, U.S. Department of Health and Human Services, Research Triangle Park, NC; NTP TR 453; PB 96-3369.

Oller, A.R., Kirkpatrick, D.T., Radovsky, A., Bates, H.K. (2008) Inhalation carcinogenicity study with nickel metal powder in Wistar rats. Toxicol Appl Pharmacol, 233(2): 262-275.

Oskarsson, A., Tjalve, H. (1979) Binding of 63Ni by cellular constituents in some tissues of mice after the administration of 63NiCl2 and 63Ni(CO)4. Acta Pharmacol Toxicol (Copenh), 45(4): 306-314.

Oskarsson, A., Andersson, Y., Tjalve, H. (1979) Fate of nickel subsulfide during carcinogenesis studied by autoradiography and x-ray powder diffraction. Cancer Res, 39:4175-4182.

Pott, F., Ziem, U., Reiffer, F.J., Huth, F., Ernst, H., Mohr, U. (1987) Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats. Exp Pathol, 32(3):129-152.

Pott, F., Rippe, R.M., Roller, M., Csicsaky, M., Rosenbrunch, M., Huth, F. (1989). Tumours in the abdominal cavity of rats after intraperitoneal injection of nickel compounds. In: Proceedings of the International Conference on Heavy Metals in the Environment, 12-15 September 1989, Geneva: World Health Organization. 2: 127-129.

Pott, F., Rippe, R.M., Roller, M., et al. (1992) Carcinogenicity of nickel compounds and nickel alloys in rats by I.P. injection. In: *Nickel and Human Health: Current Perspectives* (Eds. Nieboer, E., Nriagu, J.O.). John Wiley & Sons, New York. Pp. 491-502.

Schwerdtle, T., Hartwig, A. (2006) Bioavailability and genotoxicity of soluble and particulate nickel compounds in cultured human lung cells. Mat.-wiss. U. Werkstofftech, 37(6):521-525.

Sunderman, F.W. Jr., Hopfer, S.M., Knight, J.A., et al. (1987) Physiochemical characteristics and biological effects of nickel oxides. Carcinogenesis, 8(2):305-313.

Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. (2007) Particokinetics in vitro: dosimetry considerations for in vitro nanoparticle toxicity assessments. Toxicol Sci, 95(2):300-12.

Vekaria, H., Hamirani, A., Andrade, A., Castillo, D., Scheiner, M., Chen, J. J., and Landolph, J. R. (2006) Genotoxicity of Nickel Metal Grinding Dust, Spherical Particles of Elemental Nickel, Fine Particles of Nickel Compacts, and Nickel Sulfate by Short-Term Assays in C3H/10T1/2 Cl 8 Mouse Embryo Cells. Progress Report V to the Nickel Producers Research Association (NiPERA) of July 18, 2006.

Verma, R., Ramnath, J., Clemens, F., Kaspin, L. C., and Landolph, J. R. (2004) Molecular biology of nickel carcinogenesis: Identification of differentially expressed genes in morphologically transformed C3H/10T1/2 Cl 8 mouse embryo fibroblasts cell lines induced by specific insoluble nickel compounds. Molecular and Cellular Biochemistry, 255: 203-216.

Additional References Suggested by Reviewers

Abbracchio, M.P., Heck, J.D., Caprioli, R.M., and Costa, M. (1981) Differences in surface properties of amorphous and crystalline metal sulfides may explain their toxicological potency. Chemosphere, 10:897-908.

Abbracchio, M.P., Heck, J.D., and Costa, M. (1982) The phagocytosis and transforming activity of crystalline metal sulfide particles are related to their negative surface charge. Carcinogenesis, 3:175-180.

Andersen, A., Berge, S.R., Engeland, A., Norseth, T. (1996) Exposure to nickel compounds and smoking in relation to incidence of lung and nasal cancer among nickel refinery workers. Occup Environ Med, 53: 708-713.

Christie, N.T., Sen, P., and Costa, M. (1988) Chromosomal alterations in cell lines derived from mouse rhabdomyosarcomas induced by crystalline nickel sulfide. Biol. Metals, 1:43-50.

Costa, M., Nye, J.S., Sunderman, Jr., F.W., Allpass, P.R., and Gondos, B. (1979) Induction of sarcomas in nude mice by implantation of Syrian hamster fetal cells exposed *in vitro* to nickel subsulfide. Cancer Res, 39:3591-3597.

Costa, M. (1980) *Metal Carcinogenesis Testing: Principles and In Vitro Methods*. Humana Press, Inc., Clifton, NJ, 1980, 170 pp.

Costa, M., Abbracchio, M.P., and Simmons-Hansen, J.S. (1981) Factors influencing the phagocytosis, neoplastic transformation, and cytotoxicity of particulate nickel compounds in tissue culture systems. Toxicol Appl Pharmacol, 60:313-323.

Evans, R.M., Davies, P.J.A., and Costa, M. (1982) Video time-lapse microscopy study of phagocytosis and intracellular fate of crystalline nickel sulfide particles in cultured mammalian cells. Cancer Res, 42:2729- 2735.

Heck, J.D. and Costa, M. (1982) Surface reduction of amorphous NiS particles potentiates their phagocytosis and subsequent induction of morphological transformation in Syrian hamster embryo cells. Cancer Lett, 15:19-26.

Heck, J.D. and Costa, M. (1982) Extracellular requirements for the endocytosis of carcinogenic crystalline nickel sulfide particles by facultative phagocytes. Toxicol Lett, 12:243-250.

Horie, A., Haratake, J., Tanaka, I., Kodama, Y., Tsuchiya, K. (1985) Electron microscopical findings with special reference to cancer in rats caused by inhalation of nickel oxide. Biological Trace Element Research, 7(4):223-239.

Patierno, S.R., Sugiyama, M., Basilion, J.B., and Costa, M. (1985) Preferential DNA-protein cross-linking by $NiCl_2$ magnesium-insoluble regions of fractionated Chinese hamster ovary cell chromatin. Cancer Res, 45:5787-5794.

Sen, P. and Costa, M. (1985) Induction of chromosomal damage in Chinese hamster ovary cells by soluble and particulate nickel compounds: Preferential fragmentation of the heterochromatic long arm of the X-chromosome by carcinogenic crystalline NiS particles. Cancer Res, 45:2320-2325.