The Nitrosamines as Impurities in Drugs; Health Risk Assessment and Mitigation Workshop

Moderated by Dr. Aisar Atrakchi

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9 a.m.

Food and Drug Administration (FDA)

Office of New Drugs

White Oak Campus

10903 New Hampshire Avenue

Silver Spring, MD 20903

Reported by: Irene Gray

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A P P E A R A N C E S

List of Attendees:

Dr. Gerhard Eisenbrand

Dr. Soterios Kyrtopoulos

Dr. Joseph Guttenplan

Dr. Errol Zeiger

Dr. John R. Bucher

Dr. Jerry M. Rice

Dr. Stephen S. Hecht

Dr. Richard H. Adamson

Dr. Michael DiNovi

Dr. Aisar Atrakchi

Dr. Sruthi King

C O N T E N T S

P R O C E E D I N G S

MS. PAINTER: Hello, everybody, and thank you so much for joining this two-day workshop on Nitrosamines as Impurities in Drugs. Before we get started with Day 1, we did want to just go over some house rules, just things to keep in mind as we go through this workshop.

For those who are not speaking, please be sure to keep your phone or computer audio on mute. All attendees will be muted, and only the panelists who will be speaking in today's discussion will have the ability to unmute.

As far as using the video feature, only the panelists that will be engaging in today's discussions will have their video features turned on. So, if you are not speaking, please be sure to have your video feature turned off. And regarding questions and discussions during today's workshop, please utilize the QA to submit any questions that you'd like to have answered during today's workshop using the QA feature. You may not automatically receive a response from us, but we do have a team of

moderators reviewing the questions, and then they will be submitted and addressed as time allows during this workshop.

If you are a panelist who will be speaking, please check your chat feature as the host will be prompting you when it is time for you to present.

And last, I just wanted to let everybody know that this workshop today and tomorrow will be recorded, and the slides will be made available on the FDA webpage after this workshop.

Thank you.

DR. ATRAKCHI: Can everybody hear me?

MS. PAINTER: Yes, we can.

DR. ATRAKCHI: Thank you. Good morning, good afternoon, and good evening. My name is Aisar Atrakchi. I am a pharmacology/toxicology supervisor in the Division of Psychiatry, Office of Neuroscience in the Center for Drug Evaluation and Research, CDER. I am a member of the CDER Nitrosamine Task Force and one of the organizers of this workshop, as well as the Moderator.

I would like to welcome all of you to this workshop on Nitrosamine as Impurities in Drugs. There are over 3,500 registrants for this workshop.

Over the past 2-1/2 years since the detection of nitrosamines in medicines, both the regulators and the pharmaceutical industry have been challenged with the many aspects of this public safety incident. There is a discontinuity in our knowledge of nitrosamines since most of the research and science was conducted 50 years ago. In order to make the best scientifically-based decisions on the safety and risk assessment and mitigation, we have gathered the most qualified nationally and internationally recognized scientists and researchers in this field to inform us of previous foundational knowledge and the current state of the art practices.

We have prepared a number of critical questions for the panelists to discuss and answer over the next two days. Before we begin the presentations, I would like to remind everyone this is a scientific workshop. No policy or regulatory comments will be discussed. So, please limit your questions to the

science and to the discussions. You may send a clarifying question as just mentioned to you or comment to the chat box or the Q&A box, and we will attempt to answer as many as possible during the discussion of the particular question or at the end of the day. However, please note that the objectives of this workshop are for the experts to discuss and deliberate on the questions the Agency has provided to them.

Without further delay, we begin with the first presentation by Professor Gerhard Eisenbrand to give us an overview on the Chemistry and the Toxicity of Nitrosamines to set the stage.

This will be followed by a presentation by my colleague Dr. Sruthi King, who will provide the background on the Nitrosamine Contamination Incident in Drugs that was identified in June of 2018.

Biographies of all of the panelists have been posted on the FDA website for your information, and as also mentioned earlier, this workshop will be recorded and will be available soon after the workshop.

And with that, please, we will begin with Dr. Eisenbrand. Thank you.

DR. EISENBRAND: Thank you very much, Dr. Atrakchi. And I may start with stating that my presentation reflects my personal views as a retired professor of fruit chemistry and toxicology, and I may admit it may be perhaps be biased a bit by my scientific experience in the history of field of N-Nitroso chemistry and biology for the last 50 years or so. So, for some time the subject has been thought to be adequately explored or for some people, even over-explored, but I myself never shared this opinion, mainly because there are definitely knowledge gaps, especially with respect to the problem of in-vivo N-nitrosation.

Now it has resurfaced as a consequence of discovering seemingly unexpected drug contamination originating from changes in a production process introduced without awareness of the risk to generate nitrosamine contamination. This exemplifies the need of adequate safety checks of processes based on scientific knowhow. Now, after realizing that this

appears to be quite a general problem, we are faced with increasing complexity with respect to the potential causes. There is another point. And as I will outline, the second field of complexity is connected to the fact that human physiology provides ample potential for endogenous formation of nitroso compounds. And this needs to be evaluated as well when assessing respective health risks occasionally associated with APIs carrying the risk to become nitrosated.

Now, could I get the next slide please? This slide just shows the discovery, history, and earlier research. There have been acute intoxication reports, several. The first one was by Freund who described clinical manifestations and studies of acute human intoxication resulting in parenchymatous hepatitis, and this was later on almost -- or even much later then it was again taken up. And toxic properties of dimethylnitrosamine were described because dimethylnitrosamine had been proposed at that time as an industrial solvent. So, it turned out for those two, Dr. Barnes and Magee that the intoxication

symptoms, rescinded to some extent intoxication with pyrrolizidine alkaloids from which it was known already that these were hepatocarcinogens. So, it was very logical to see whether nitrosodimethylamine might also be a hepatic carcinogen or a carcinogen all together, and this was more or less against the current views at that time because this was a very well water-soluble and very low molecular weight compound contrary to all the other known carcinogens like polycyclic aromatic hydrocarbons and so on.

But to cut a long story very short, this indeed has been shown to be the case, and this later was repeated by a German group, and these authors also tested the next analog, that was dimethylnitrosamine, showed that it even was more carcinogenic. And then within a relatively short time, the group of Peter Magee showed that dimethylnitrosamine was a methylating agent, methylating DNA very effectively.

Next please. So, again cutting a long story short, there was then a tremendous amount of research, biological and chemical. And I just

mentioned two publications that reflect the results of all this research. One is what was long called in German The Nitrosamine Bible that was this publication here as structure activity related and dose response related, very extensive investigation of 65 N-nitroso compounds published in 1967. And then another time John showed the megamouse rodent studies that have been evaluated very formally by the group of Peto and his coworkers with a very detailed dose response, details especially for dimethyl- and diethylnitrosamine.

Next, please. So, to get a summary of the biological activities of NOC shows NOC means N-nitroso compounds. It shows that over 90 percent, more than 300 nitroso compounds are known to be carcinogenic and in animal experiments, so if you find a new compound or a new structure, the probability is relatively high that this may be a carcinogen, but there are some structural activity showing that one can get a little bit more power into the predictions of carcinogenic potency. The most investigated compounds, dimethyl- and diethylnitrosamine and also

the tobacco-specific compounds use tumors in a very wide spectrum of animal species up to subhuman primates, and there is no species that is found to be resistant. Structured community studies have helped to conclude on structural elements that are responsible for carcinogenicity, and those vice versa that may abrogate carcinogenicity. And there is a characteristic feature of many of these nitroso compounds that they may induce at the right dosage quite specific organotrophic activities. Almost all organs of experimental animals that have been used are listed here.

The last one is that the bioactivation of nitroso compounds that the interaction is crucial induced in targets proceeds basically similar in animals and in human tissues.

Next please. So, the metabolism is very well investigated, and it shows that the most critical event is the alpha-C hydroxylation of nitroso compounds. Nitrosamines in this case where you see that after the alpha-hydroxy group has been introduced by cytochrome p450, then the enzymes, and aldehyde is

split off, and you get an alkylating intermediate, either diazonium ion or a -- cation, which then is able to alkylate nulceophilicites in the DNA at different DNA bases, including also phosphates by the way. Metabolism is not necessary for the direct-acting nitroso compounds like here shown, the alkyl nitrosureas that just alkylate by direct decompensation, often catalyzed by basic media.

Next please. So, there is a group of rules concerning prediction of carcinogenic activity from structures. Since the alpha-C hydroxylation, the metabolic one matters so much that it is easy to conceive that if you put in branching in the other position, you will inhibit or at least decelerate the metabolic activation. That is definitely the case of branch compounds, and the alpha position branch compounds are less carcinogen than the stretching ones. And this goes up to the point that you have tertiary butyl branch. They are noncarcinogenic.

The second point of consideration also is that if you have a tertiary butyl substituent in this position, then you very often have also a slowing

down of nitrosation because it leads to some extent the nitrosation.

Next slide. So, compounds that are known to be relevant for human exposure versus nitroso compounds are shown here in this selection. There are volatile compounds. This has this reflection in the analytical determination of these compounds that are basically determined by a purification at the destinative step. Dimethylnitrosmine up to nitrosomorpholine. These are the carcinogenic or even highly carcinogenic ones. Nitrosopyrrolidine is somewhat less active than the short chain ones, nitrosomorpholine, and then there are non-volatilized nitrosodiethylamine known to be a contaminate of cosmetics, or they have been a contaminant of cosmetics. And then the nitrosoamino acids, most of them are noncarcinogenic with the exception of nitrososarcosine that is a weak carcinogen. The reason for this non-carcinogenicity is believed to be the very good water solubility and the ionization under physiological pH, and this is also true for compounds that may contain amino groups, ionizable, or

protonable amino groups that may then if the compound is protonized also inhibit or at least make this option quite slow.

Next slide. So some words to the basics of formation of N-nitroso compounds.

Next please. So, this depicts -- it is a busy slide, but it depicts more or less what has been published already in 1975 by Sid Mirvish and depicts rates and the factors that are important for the rates. First of all, it is important to realize that the nitrosating agent itself is N203 in aqueous acidic solution, and the formation of this N203 is going through the interaction of two molecules of undissociated nitrous acid. So, the formation of nitrous acid is favored by proton concentration. The more acidic the milieu is, the more nitrosating agent is available. On the other side, it is only the unprotonated amino nitrogen that can be nitrosated, so in other words, the results is then this bell-shaped curve you see on the righthand side of the panel that shows the pH dependency of the nitrosation rates in short.

The second point also to take into consideration is that the pK(a) value of the amines very strongly determines the nitrosation rates, saying that high pK values, strongly basic amines are relatively much less easily nitrosated than weakly basic amines. The examples shown here are dimethylamine for a strongly basic one and for instance morpholine or piperazine for a weakly basic amine.

Next please. This is also shown again here. On the left-hand side, you will see what you have seen directly before, but on the right-hand side, there is a collection of data showing that this in vivo nitrosation can be shown interactions by giving appropriate dosages of amines, not nitrosamines but amines, and of nitride. And in that case, the green field here, the green area, shows the high pK(a) amines that are not producing enough nitrosamine in the acidic medium of the gastric milieu of the stomach, whereas the ones that are within this red field here, the low pK(a) amines, the weakly basic amines, they have been shown like nitroso

methylbenzylamine or nitroso piperazine, they have been shown to yield enough nitroso compound in vivo, in the stomach to induce the same tumors as what would be seen when getting the nitroso compound itself. So, that's the first information about this in vivo situation that has been tested many years ago.

Next please. So, as another point of importance, and that is if you remove the possibility of the amine to get protonated, for instance by interaction with formaldehyde, then the nitrosation is no longer dependent on the acid medium, but it can also directly go on in neutral or either basic milieu, and that is a problem that has been faced mainly in occupational exposure situations. For instance, with the metal industry, the cutting fluids that are normally weakly basic. Where nitrogen oxides in the industrial environment directly interact with amines and form nitroso compounds, and this may also happen with nitride.

Next please. So, to get this together, I have not mentioned yet the primary amines that also to some extent might directly react in an acidic

medium for instance to form diazonium ions, so in other words, they may also form electrophiles that could be available for interacting with DNA for instance or biological material. However, the rates are much slower than those for secondary amines, which normally nitrosate quite rapidly under the conditions I have just outlined before. And of course, as mentioned in basic or nonaqueous media, there is also a rapid possibility of rapid interaction with nitrosating agents, since the protonation is not completed. Most tertiary amines form also nitroso compounds by a process called dealkylating nitrosation. Normally, a few significant exceptions at much smaller rates, and then they have the catalysts, formaldehyde, and other carbonyl compounds that may interact also. Halogenides and thiocyanate for instance are acting as catalysts, whereas compounds that are scavenging nitrosating agents, like ascorbates, tocopherols, and phenolics, for instance flavonoids, they are normally considered as inhibitors of the nitrosating reaction.

Next please. Again, quite a busy

slide, but it's important. This is a relevant tertiary amine. It is nicotine. Nicotine undergoes quite easily nitrosation, dealkylating nitrosation, and in response to the ring opening reaction or demethylating reaction that is going on, we get three different compounds that are shown here, NNN, NNA, and NNK, which is very involved in tobacco-specific nitroso compound.

And the lower part of that slide shows the metabolic activation, which is well known and very well investigated, methylating or into a ketobutyrating agent that interacts with the DNA. Finally, and on the utmost right-hand side, you will also see a reaction that might be called a detoxification reaction because the keto group undergoes to some extent partial reduction to the alkyl ion, and the alkyl is then excreted in the urine as the corresponding glucuronide. Now, NNK and NNA have been rated to Group 1 carcinogens by IRC. So, they are carcinogenic to humans because there is sufficient evidence for carcinogenicity in animals, and there's strong mechanistic evidence also in

exposed humans. And there is another aspect that also is quite important for human exposure, and that is --

Next slide, please. That we also have passive exposure to tobacco smoke, very well investigated by my dear colleague, Stephen Hecht, who has been working very intensely on these subjects. And the base of the biomarkers excreted in the urine of nonsmoking, exposed people, starting with transplacental exposure already and measured for instance in urine of newborns and other exposed population that may be passively exposed to so-called secondhand smoke. You see that it is about 1 to 5 percent of those in smokers as being rated there. So, it is quite significant.

Next slide please. So, let us just have a short walk-through nonfood products, cosmetics as the first example, and personal care products have been found many years ago already that these are providing exposure maybe by nitroso-methylalkylamines and some others that are listed here up to nitrosomorpholine and long chain components. The main problem there was insufficient purity of basic

materials. In other words, it has been so rapidly thereafter when people became aware of this problem, and also there have been some nitrosating preservatives like bronopol or bronidox that are transnitrosating or nitrosating agents.

Next slide, please. So, it's clear that mitigation based on the knowledge was quite effective. Purity was determined by purity specifications, maximum content of nitrosamines. And these all ended finally up an estimate of well-known exposure, systemic exposure by dermal application of cosmetics lower than 0.05 micrograms per person per day.

Next, please. One short word to the occupational exposure also known since many years. One of the courses I have already alluded to is the use of nitride as a corrosion inhibitor or the interaction of nitrogen oxides in an industrial situation with the amines that are present in cutting -- for instance in metal cutting fluids. And this contamination went up to PPN, even high PPN values.

Next slide, please. Again, the

logistics of mitigation are just written here. They are spelled out in the Technical Rules for Hazardous Substances. The last edition I think is 2018 in Germany or whatever it is called, TRGS 522. So, these are the mitigation measures recommended. No use of nitrite as a corrosion inhibitor, using nitrosation inhibitors, and replace chemicals giving rise to carcinogenic nitroso compounds by those that do not give rise to carcinogenic compounds. And that principal or that strategy has been called the strategy between "safe amines."

The next slide shows -- I think an example for that you see on the left-hand side compounds that are used, for instance, in rubber industry for vulcanization of rubber. These are thiuram disulfides, and they under good protection conditions, they would then generate dimethylnitrosamine or nitrosomorpholine at the lower example case. And on the right-hand side, you'll see what we can do for prevention or mitigation. These compounds can also equally be used or almost equally be used for technical purposes, and that falls along

carcinogenic nitrosamine within that green circle, and the same is true for the lower line examples shown here. Alpha branch nitrosopiperidine or N-methyl-nitrosopiperazine, which is not known to be carcinogenic contrary to dinitrosopiperazine, which is quite a strong carcinogen. Okay. So, that is the example of safe amines.

The next, please. We go now to the technical rules a little bit more in detail because I want to show you regulations as written in the TRGS 552. There is a tolerance and acceptance concentration. For instance, in the air, that is being inhaled at working places. At the moment it is 0.75 micrograms per cubic meter is the tolerance and one-tenth of it or 0.75 micrograms per cubic meter is the acceptance concentration also, not only for the individual nitroso components but also for the sum of it if there are several components in the air found.

Next please. Now the last word on the exogenous is exposure to food. They have the nutrition exposure. Interesting to see, but the very first incidence reported in the literature was in an

animal meal based on fishmeal that had been treated with nitrite. In that case, hepatotoxic factor, which at that time was not known, has been identified and later more or less identified because it was a very high contamination that it was dimethylnitrosamine, and truly enough the animals got liver toxicity. So, this, of course, triggered almost an avalanche of research into foods because of course animal-based foods are very often cured or smoked or at least should be an expected formation of nitroso compounds, and this research was long hampered by the relatively insufficient analytical methods. Finally, developed the thermal energy analyzer (TEA) and later on of course, and that is the present state of the art, is the coupling of chromatographic separation methods with multiple mass spectrometry for identification.

Next please. So, the processing methods -- because nitroso compounds in foods are process-related contaminants, which are potentially responsible are curing with nitrate or nitrite. So, reduction of these could help. Then, the addition of literature, then for smoked food, lowering of the NOx

content in the smoke with which foods are treated. Drying or kilning of malt by direct firing techniques has been found to be shown to nitrosamine formation. And in rare cases also packaging by migrational nitroso compounds into the food.

Next please. So, one example here is a kiln where barley after germination is being dried in a kiln. And also some specific browning reactions. And one can directly see whether the direct firing techniques with burners of above 1,100 degrees Celsius, they produce a lot of nitrogen oxides, and these are swept through the malt and of course interact then with constituents in the malt to form nitroso compounds, in other words to limit the temperature to degrees or to use indirect firing techniques or heating techniques that are very well established. Indirect heating techniques used throughout centuries that would remove that contamination quite efficiently.

Next slide. The main precursor for nitrosodimethylamine or NDMA in barley is gramine, again a compound that can easily be interacting with

nitrogen oxides by splitting off the nitrosamine elements. And as I said, the mitigation measures were quite effective to reduce the contamination to really very low levels nowadays.

Next, please. I think shows the estimated daily dietary intake of nitrosodimethylamine. And this is a collection that has been published by Hrudley up here in 2013, but the data are quite outdated I would say. So, there is not very much current data on nitrosamine contents in food nowadays. I think this is important for several reasons that we get updated data on this exposure for almost unavoidable food consumption of course, almost, but certainly unavoidable, almost unavoidable for the nitrosamine exposure that is connected to it. So, you see, this is all below 0.2 or 0.3, except for instance, for Australia. But as I said, I think we definitely need updated data on this exposure situation.

Next slide. So, now we turn to the endogenous formation thematics, and I start with mentioning the pioneers, not only for endogenous

formation but also for interaction of compounds with nitrosating agents. Sander and Burkle had already as early as 1969 made the first experiments to show that a secondary amine together with nitrite given to animals by gastric tube induces tumors that are indiscernible from the tumors of correspondent nitroso compounds. So, there is situ formation in the gastric compartment of nitroso compounds that is responsible. Then I have to mention Willie Lijinsky who had a tremendous amount of studies concerning the interaction of drugs with nitrous acid as a source of carcinogenic nitrosamines, incredible work and very important to revisit because in the face of our current situation. Finally, Richard Loeppky is also a very important contributor because he has been mainly elucidating with formation of nitroso compounds from tertiary amines, also a very important piece of science that is published in many publications.

Next, please. When we consider endogenous formation, we need to take into consideration that we have already concerning the upper gastrointestinal nitrite, we have a situation of

circulation. As soon as you take up nitrite, for instance, by consuming nitrate-rich vegetables, then this process will go on, which is resorption from the gastrointestinal tract, and then that circulation, but then nitrate is resecreted through the salivary glands back into the mouth, the cavity of the mouth. The mouth has its own microbiome, and in that microbiome, there are microorganisms that are able to reduce the nitrate to nitrite. In other words, generates part at least of nitrosating agents by this way. There is about 25 percent of a given dose of nitrate that is recirculated, and about 6 percent of it that has been very well studied is reduced to nitrite. So, there is a potential already here. But that is not he only one. The other one as many of we know.

Next please. Because there is an interrelationship between nitrate, nitride, and nitrogen monoxide. The first point is that very researched by Steven Tannenbaum's group showing that humans produce daily about 50 mg of day for a 70 kg person, endogenous synthesis of nitrate that has been verified by input and output, and, of course, there is

a lot of variety of enzymes and proteins that can act as reductases and reduce nitrate via nitrites to nitrogen monoxide. There is further, of course, the function of nitrogen monoxide as a signaling molecule, which is generated from arginine and also creates a sustained source for nitrogen oxides and from that on, then also nitrite and nitride. And there is also a key component that is found in response to bacterial infections and during inflammatory reactions. Various publications address this. To give just a number for the dietary nitrate uptake, that is an average about 175 mg per day as stated by EFSA (European Food Safety Agency) in 2008. This interrelationship of nitrates, nitrides, and nitrogen monoxide is again shown in the --

Next slide, please. These three components are metabolically interconvertible. Nitrogen monoxide can be oxidized to nitrate and nitride, and vice versa, nitrate and nitride can be reduced to nitrogen monoxide.

So, in summary, we have a certain endogenous physiological potential of generating

nitrogenous agents that is in part dependent on the nutritional exposure but is regulated and influenced by many other physiological parameters.

Next please. Of course, there have been estimates of endogenous formation of nitrosodimethylamine. I should perhaps mention before I go into that, that the urinary excretion of nitrosated amino acids has been used for a long time as an indicator, a biomarker for endogenous nitrosation in humans, and that is possible because these nitroso compounds are not mutagenic, not carcinogenic, and they are practically quantitatively excreted in the urine. So, they can be used as exposure biomarkers for nitrosation in vivo. And to go through carcinogenic nitroso compound, for instance, N-nitrosodimethylamine, this is very difficult because nitrosodimethylamine has a very short halfway in vivo. It is rapidly cleared from the body, maybe by cytochrome P450 metabolism. And therefore, attempts to measure this are sort of really difficult. And it is not only the difficulty itself you see, the data of these measurements are quite old,

1993 up to '86. Because there is another aspect, which is also important, and that is that in those early days, very often nitrosamine analysis has been plagued by the formation of artificial formation caused by artificial formation of nitroso compounds during workup and analysis. And if you see results in the literature that do not completely prove, for instance, by the addition of releasing the nitrosatable tracer compound that artificial formation of nitroso compounds is prohibited or inhibited, then these results are normally not really trustable. Anyway, if one takes these earlier results of very low levels of NDMA in blood samples that have been considered as reflecting steady state, then one would have incredibly high amounts of endogenous exposure to this compound. As you see here, it would be up to 2,500 mcg per day or 1.4 to 35 mcg/kg per day. And if one bases this on a biomarker, the biomarker of alkylation of O6 oxygen of guanine that is also very well developed as a technique. Then, you would end up in a similar range of about 18 mcg/kg per day. I think these are important points to mention here and

to show that we really need definite, dependable confirmation and delegation of these results. I think it is very important to know more about this.

Next, please. Before we come to the last part of endogenous nitrosation considerations, just I would like to mention just shortly the WHO nitrosation assay procedure or short NAP assay that has been published very early already in 1980. This is a very simple chemical test under rugged conditions with relatively high concentrations showing the reactivity as is seen here on the left and on the righthand side of a couple of compounds that have been investigated by this test. You see that secondary amines of course are very high on the scale but also tertiary amines on the other side. And the problem with this test was that there were never cutoff levels of reactivity really defined scientifically. So, it has not been used very much.

Next, please. Human information of formation of nitroso compounds in humans is of course available. There are several publications on this one. One of several publications. This is one by

Tricker and Preussmann showing in patients with parasitic infections that piperazine is nitrosated endogenously, and it can be measured by urinary excretion of mainly mononitrosopiperazine, a little trace of binitroso and the corresponding metabolites. And the other compound mentioned is amidopyrine because it is an extremely reactive compound towards nitrosating agents, almost considered as a reagent to show the presence of nitrosating agents. And here, in this case, the in vivo nitrosation was measured in urine on simultaneous passage of some ethanol to inhibit cytochrome P450 clearance. And then it became available in the urine, nitrosodimethylamine could be measured.

Next, please. So, the example of amidopyrine and its close analog, metamizole. The amidopyrine as I mentioned is extremely reactive and responding with the formation of dimethylnitrosamine, the other part of the panel. And this resulted very soon in withdrawal of amidopyrines from the market. And the analog here is metamizole. That is still on the market because it has been shown that the nitroso

compound that is shown, this mononitroso compound here shown in the green circle is nonmutagenic and noncarcinogenic. Again, close analogs showing vastly differing properties.

Next, please. Then, of course, you are all quite familiar with the occurrence of formation of dimethylnitrosamine in Sartans in the dimethylbiguanide (metformin) that is shown here. To my knowledge, at least, the source for the NDMA formation is not really elucidated yet. But this was, as I mentioned at the very beginning, the change with the Sartans, the change in the production process to a solvent, dimethylformamide, which of course then can react with nitrite that has been used to quench and destroy the azide that had been used to speed up the tetrazole ring. This then was the cause to form dimethylnitrosamine.

Next, please. That is similar for ranitidine, again structured as you may see here. The dimethyl amino group attached to the furan ring system. One could predict that this will carry easily. What I think is much more important is the

published study recently in 2016 with human volunteers where it was shown that enhanced levels of dimethylnitrosamine were excreted in the urine. And analytically, this was all right because these authors not only had the best sophisticated instruments, instruments with mass spectrometry to measure, but also, they were keen to shown that there is no artificial formation during analysis. So, from an analytical viewpoint, this is, in my opinion, all right. And again, this would be quite substantial, that is a nitrosamine formation rate that is going on as measured in humans. If it can be confirmed, in my opinion, it is very important to confirm, especially also raises the question why urinary excretion of dimethylnitrosamine is so relatively high because as we know, normally, it is very rapidly cleared and eliminated by metabolism.

So, we have one more, which is another H2 receptor antagonist. Please, next slide. That was the first one to study cimetidine. That is the one here. That can also be easily nitrosated, but unexpectedly this compound, although it was mutagenic,

it was revealed not to be carcinogenic. And the group Magee has contributed a lot to show that the mechanism is that this compound is metabolically mainly denitrosated by glutathione and glutathione transferases by other SH groups of cystine or hemoglobin groups and even by cytochrome P450. And they also realize that the imidazole ring here may also be ionized, so this would also contribute under physiologic conditions to keep perhaps the nitrosatable ability or the biological effect of the nitroso compound. That is all I have to say. So, we come to the end.

Next, please. Most of you will know the group limits that have been defined recently based on the TD50 values of the original Gold Database, now the Lhasa Database that came to either lower default values, which are, you know, much lower than those that might be seen either by food exposure or even by endogenous exposure if these values can be confirmed.

Next, please. This should show the breakout. As I promised, I would like to contribute to show open questions, knowledge gaps, and research

needs. First to the exposure, I think it is very important to get a database update on the exogenous exposure. I think maybe from diet. Because it may be used as a suitable reference, correct, at least the suitability needs to be considered for risk assessment of other exposure pathways like contaminated drugs. And of course it is even more urgent to come to grips with the endogenous exposure and to develop validated analytical methodology to use PBBK-based estimates for human endogenous exposure and to check the productivity of the biomarkers of in-vivo formation. Are the nitrosamine acids also predicting carcinogenic nitroso compound formation are all important questions. And for the mitigation, the most important point, of course, is to scrutinize the technology and the processes for drug production to be sure that one can really mitigate interactions of potential nitrosating agents with APIs. But I think one should not totally leave out the possibility that given a pharmacological or toxicological tolerance of an API, that one can explore possibilities to replace critical structural elements as successfully achieved in other

areas. With these open questions, I leave you now, and thank you very much for your attention. Thank you.

MS. KING: Good morning. My name Sruthi King. I am one of the Associate Directors of Pharmacology and Toxicology in the Office of Generic Drugs in the Center for Drug Evaluation and Research. I'm one of the members of the Safety Team on the CDER Nitrosamine Task Force. We have been working together for the past 2-1/2 years, and as we head into the technical discussion today, my objective is to provide some context into the considerations and strategies used by FDA since the start of this incident and to highlight some of the ongoing challenges from the scientific and regulatory perspective.

Next slide, please. So, this is just to indicate that the views presented today are mine and do not reflect FDA policy.

Next slide, please. As you have just heard Dr. Eisenbrand present, he gave an excellent introduction to nitrosamines. We know that nitrosamines are present in food, water, tobacco,

multiple sources in our environment. We know that their chemistry is not new. Their toxicity and potency is not new. We know that there are potent rodent carcinogens, and some are probably human carcinogens. However, the presence of nitrosamines in drug products was alarming when we first became aware of it at the FDA in 2018. This contamination incident affected products globally, resulting in recalls of vital medications. And this required the development of highly sensitive analytical methods to detect and quantify these nitrosamines and investigate the root cause of formation of these compounds, so that we could identify appropriate control strategies. Managing this nitrosamine contamination incident in drug product has required multidisciplinary approaches to conduct the risk-benefit assessments, to collaborate with industry, and with our international regulatory partners, and to develop effective communication strategies so that our patients are aware of what is in their drug products.

Next slide, please. So, CDER (Office of Generic Drugs) became aware of the presence of

N-nitrosodimethylamine or NDMA in valsartan, which is one of the angiotensin II receptor blocker class of drugs. Since that time, we have learned that nitrosamines have been identified in active pharmaceutical ingredients or APIs, along with finished drug products. And this contamination of nitrosamines has been seen in generic drugs, as well as brand drugs, although the effect has been greater on generic drugs. Multiple nitrosamines have now been identified, and so we are looking into control strategies for single and multiple nitrosamines in a drug product. What you see on the righthand panel of this slide is some of the nitrosamines that FDA has identified in drug products and posted acceptable intake limits. Also included in this list is 1-methyl-nitrosopiperazine and 1-cyclopentyl-nitrosopiperazine, which have been identified in some anti-infectives. Despite the nearly 2-1/2 years into this contamination issue, we have still many ongoing challenges.

Next slide, please. So, what are some of the considerations that we make when we become

aware of the presence of a nitrosamine. We first look at whether it is a single or multiple nitrosamine. Is it a risk of formation, or are there actual levels being detected? Are the analytical methods being used sufficiently sensitive? And what is the root cause investigation tell us? Is this an API issue, or is this a drug product issue, or is it both? Once we are aware of what is the specific nitrosamine, we then consider what are the available nonclinical data to establish an acceptable intake. We then also consider what are the products that are being affected? What is the patient population that is being impacted by this contamination issue? Are these products medically necessary? What are the levels detected in the actual drug product, and how does this correspond to the acceptable intake of that nitrosamine? Should products be recalled, and if a recall is required, will this precipitate a drug shortage if there are no alternate options available. Therefore, there are multiple considerations that go into managing this issue and also to determine what is the appropriate next step.

Next slide, please. This slide summarizes some of the key events and timeline of this incident from FDA standpoint. So, for each of the products that have been impacted by the nitrosamine contamination, FDA has conducted a risk assessment and posted acceptable intake limits, along with appropriate analytical methods for that nitrosamine and issued communications related to the risks of exposure, along with recalls that have happened, so that stakeholders are aware. As the incident evolved, FDA published a guidance in September of 2020 on the control of nitrosamines and drug products. We are actively engaged with our key stakeholders and researchers, along with our international regulatory colleagues to identify best approaches for risk assessments and control and mitigation strategies. As you can see, the incident began with contamination issue in antihypertensives in the ARBs, the angiotensin II receptor blocker drugs, and has now encompassed many classes of drugs, including ranitidine and nizatidine medications to manage diabetes and also infectious diseases such as

tuberculosis.

Next slide, please. When FDA first became aware of this contamination issue, our Center Director at the time, Dr. Janet Woodcock, activated the CDER Nitrosamine Task Force to manage this incident. She foresaw the potential broad impact on the qualify of medications and their impact on patient safety. The Nitrosamine Task Force is managed by the CDER Office of Counter-Terrorism and Emergency Coordination or CTECS. And this is a group that meets regularly. At any given time, there is over a hundred subject matter experts from across CDER and FDA that meet regularly to discuss and propose recommendations to mitigate the risk of nitrosamines in drug products and manage patient access to critical medications. As part of this effort, we routinely update senior management and discuss product and policy issues. We also engage international regulators to discuss harmonized approaches for addressing nitrosamine contamination on topics such as risk assessments, marketing actions, and sharing information related to these topics, along with communication strategies.

Next slide, please. This slide shows the Multidisciplinary Coordination that has been happening in order to manage this nitrosamine incident. In the middle, you will see the CDER Nitrosamine Task Force, and they regularly engage various groups within FDA. And this kind of multidisciplinary coordination is necessary to manage this incident locally and engage with international regulatory partners to address this global issue. I will go into further detail on some of the interactions that happen in the coming slides. But to briefly highlight some of the key interactions, the Office of Pharmaceutical Quality, chemistry experts play a critical role in the root cause investigations and analytical method development, sample testing, and managing of applications and setting expectations for pre- and post-marketing issues. Depending on the nitrosamine that is identified, pharm tox experts within Office of Generic Drugs and Office of New Drugs are called upon to identify specific acceptable intake limits based on animal data. These acceptable limits are then used to develop methods and identify

analytical targets. Clinical experts also work closely with pharm tox and quality and identify appropriate maximum daily dose for the proposed product as the maximum daily dose is used to set control limits for specific products as some products may have multiple indications. Also closely involved is the Drug Shortage Staff. When medically necessary products are impacted, Drug Shortage Staff informs us about drug supply issues. There are also Compliance experts that are involved in inspections and recalls and also managing regulatory discretion issues along with regulatory policy, Regulatory Affairs Staff that respond to inquiries from citizens. We have had Congressional inquiries into this issue. There is Post Marketing Surveillance Staff that characterize risk of exposure from post-marketing data. And then finally, there is Communications Staff and Patient Engagement Staff, and we have had to develop a robust communication plan in order to communicate to patients and stakeholders on what is happening, how FDA is managing this issue. And lastly, FDA Researchers are also actively working on developing key specific

specialized methods for quality assessments, along with nonclinical information, and nonclinical methods to optimize study conditions.

Next slide, please. So, what are some of the complexities that we have had to deal with? Root cause investigations were critical to identify what is causing formation of nitrosamines, and this would help inform control strategies. We know that nitrosamines can be formed because of process-related issues with starting materials or the API itself, intermediates. There are supply chain issues that were identified where use of recycled or recovered materials was introducing contamination into the synthesis. There are also product stability issues where excipients in the formulation for example were contributing to the formation of these impurities.

Highly sensitive methodologies, analytical methods were necessary in order to identify and quantify these nitrosamines. Sample testing was necessary to identify which of the lots consisted of nitrosamines that were above acceptable intake, and this was used to inform recall decisions.

Unacceptable intakes and sensitive methods were necessary in order to set controls within the manufacturing process.

Lastly, we had to establish risk assessment expectations where nitrosamines in pending and approved products as there could be different considerations that were necessary. From a safety standpoint, we were aware that nitrosamines are part of the cohort of concerned group of compounds, and so they needed tighter control because they posed greater risk than other compounds. We know that lifetime exposure is calculated based on an increase in one case of cancer in 100,000 patients, and this was considered an acceptable level of risk. And so our task was to balance the risk of exposure to nitrosamine versus the risk of no access to medically necessary drug. We know that potency of nitrosamines varies across compounds. Some are mutagenic and carcinogenic, while others are not mutagenic but are still carcinogenic. Also, mothers are weakly carcinogenic. There is general agreement across regulatory bodies that nitrosamine should be avoided

or tightly controlled if they are unavoidable in drug products. When we were faced with various nitrosamines, we had to determine an acceptable level, and this was done using approaches in the ICH M7 guidance, and this acceptable intake informed the analytical sensitivity of the methods that were necessary for detection of these compounds, along with informing recall decisions.

Next slide, please. So, how did we calculate acceptable intake? We had to identify a TD50, which is the dose that produces tumors in 50 percent of the animals in a dosing group from an animal carcinogenicity study. As you have heard in Dr. Eisenbrand's talk, there is a wealth of carcinogenicity information for many of the nitrosamines. The acceptable intake is the daily dose of a nitrosamine when taken over a lifetime that represents a risk of one additional case of cancer in 100,000 patients. How do we select the appropriate study when selecting a TD50. We look to see how robust the data are within the carcinogenicity study. And some of these criteria are listed here. How many

animals are there in a dosing group? What is the treatment regimen and dosing frequency? There are several nitrosamines with a number of studies that are available. Which is the appropriate study? Toxicology assessments and data presentation was another factor, along with relevance of the species and tumor. When establishing an acceptable intake, pharm tox experts in OGD, Office of Generic Drugs, worked closely with pharm tox experts in the Office of New Drugs. As I mentioned, generics and brand drugs were equally impacted by the nitrosamine issue, and therefore this collaboration was necessary to establish an acceptable intake.

Next slide, please. Not all nitrosamines have robust carcinogenicity data in the literature. Some nitroso compounds have no data at all. And so, in these cases, it was necessary to consider surrogate compounds to establish an acceptable intake. This is an approach that is described in ICH M7 where structurally or closely related structures could be used to justify an acceptable intake. When an appropriate surrogate is

not identified, we refer back to the acceptable intakes of NDMA, which is N-nitrosodimethylamine and N-nitrosodiethylamine to identify an appropriate, acceptable intake for the nitrosamine compound of interest. When we do have options for surrogates, we consider the robustness of the data that is available for that surrogate compound and structural similarities between that surrogate and the compound of interest. Just to note that some of the nitrosamines listed in the FDA Guidance have acceptable intakes that were developed using a similar process.

Next slide, please. What are some of the additional clinical complexities? A wide range of products have been affected, and this has impacted large numbers of patients with serious medical conditions, such as hypertension, diabetes, heartburn, tuberculosis. So, lack of medication, lack of medically necessary drugs could lead to public health emergencies, for example. So, the maximum daily dose is something that is necessary in order to calculate an acceptable intake or set control limits for a

specific drug product. And this is necessary to facilitate risk assessment of the manufacturing process. Another piece of this clinical risk-benefit assessment is a medical necessity evaluation. It is important to maintain patient access while balancing the risk of exposure to nitrosamines. And this multidisciplinary coordination goes into informing whether a product should be recalled. As I mentioned, prior to recall, there is a consideration of whether there are alternate therapeutic options for patients, whether the recalls will precipitate a drug shortage. And so, when there is a potential for drug shortage, additional strategies need to be considered.

Next slide, please. One of those strategies is the use of interim acceptable intakes. When patient access to drug is deemed medically necessary, FDA has applied flexibility by using interim acceptable intakes. Industry is a key partner in this short-term strategy as it offers flexibility to maintain patient access while process changes are instituted to remove or reduce nitrosamine formation. However, this requires multidisciplinary discussion

and consensus. Pharm tox staff along with clinical experts in Office of New Drugs and Office of Generic Drugs, Office of Pharmaceutical Quality, Drug Shortage Staff, Office of Compliance, and many others are needed in order to determine whether an interim acceptable intake can be tolerated to maintain patient access. This approach has been applied in several cases to mitigate drug shortages. For example, losartan was one of those cases where an interim acceptable intake was applied, along with rifampin and rifapentine.

Next slide, please. Not all products are used in the same way. Some are used as short-term, while others such as antihypertensives can be used long-term. So, how do we assess the risk of nitrosamines in short-term versus long-term use products. We know that M7 allows for adjustments based on duration of use for mutagenic impurities. We also know that nitrosamines are a cohort of concern compounds, and they are potent rodent carcinogens. Therefore, when assessing the risk of nitrosamines, we have considered lifetime exposure limits. And that is

because some nitrosamines have been shown to produce tumors at very short doses or even single doses, for instance. And therefore, there is uncertainty associated with a simple adjustment to the acceptable intake using the approach that is described in M7. In fact, M7 allows for this case-by-case approach where acceptable intakes for high-potency carcinogens, such as cohort of concern compounds, can be significantly lower than the typical less-than-lifetime adjustments. Therefore, the interim acceptable intakes do offer flexibility, but they are used as a short-term strategy to maintain patient access to medically necessary drugs. And this is a strategy we have used to avoid or mitigate a drug shortage. And adjustments based on duration of use have not been considered in determining the interim acceptable intake for a specific product.

Next slide, please. Another key factor or another key facet of the nitrosamine contamination issue in drug products from the FDA perspective has been our communication plan. Listed up here in the first bullet is the main FDA landing page for all

information associated or related to nitrosamines in medications. And using this hyperlink, you can access information that FDA has shared to inform industry of analytical methods of sampling and testing results and risk assessment strategies. This has been used to inform patients and care providers, pharmacy suppliers and distributors, list recalled products, and discuss alternate treatment options. FDA communications was critical to address media concerns and citizens petitions, along with Congressional inquiries. And finally, the Communication Staff is also actively engaged in talking with our regulatory partners internationally to discuss risk assessment strategies and harmonize on approaches on regulatory actions.

Next slide, please. One of the milestones in our communications strategy was the publication of the Nitrosamine Guidance, which you can access using the hyperlink that I provided in the first bullet. This Guidance provides detailed information on root cause assessments, regulatory expectations and risk assessments, and associated timelines, along with acceptable intakes for several

nitrosamines, and outlines risk mitigation strategies. It describes situations where there are single and multiple nitrosamines. Single nitrosamines may be allowed up to the compound-specific A1, and total nitrosamine exposure should not be exceeding 26.5 nanograms per day. There have been several webinars hosted by the Office of Pharmaceutical Quality to describe this guidance in detail to industry. Since that time, as I have mentioned, methylnitrosopiperazine and cyclopentylnitrosopiperazine have been identified, and the acceptable intake for each is posted on the FDA's nitrosamine landing page.

Next slide, please. However, we have several challenges that remain. Root cause investigations have identified multiple factors that can contribute to nitrosamine formation. We know that stability of the formulation, the excipients used, and storage conditions are some of the factors that can contribute to nitrosamine formation. Some of these factors can have broad impact on many classes of drugs because of their history of use in drug development.

The risk assessment is the key to understand whether nitrosamines can be completely eliminated or if control or monitoring are better options. In addition to working with firms that submit their risk assessments, FDA is proactively reaching out to firms with manufacturing processes that pose risk of formation of nitrosamines. The goal is to ensure that there is high quality and safe drug supply in the U.S. market. As new and more sensitive methods are developed, there is also increasing awareness of the presence of API-related nitroso impurities. These previously unidentified compounds, uncharacterized compounds, pose a unique challenge when it comes to risk assessments, and appropriate control strategies. Additionally, there is an effort to harmonize with our regulatory partners on analytical methods for testing, and to further discuss method sensitivity, monitoring, and other related topics.

Next slide, please. From a safety standpoint, it is important to acknowledge that nitrosamines are in our food, in our water, and can be formed endogenously. And so, we have to consider how

this exposure to nitrosamines from other sources, including endogenous production, how this compares to exposure from drug products. How does this impact our risk assessments and proposed control strategies for nitrosamines in drug products. In some cases, the quantity and quality of data available varies for nitrosamines. If data are not robust, identification of an appropriate TD50 to calculate an acceptable intake is challenging. We are becoming increasingly aware that improved testing methods are identifying previously uncharacterized nitroso impurities that have no published safety data. So, how do we balance this risk of exposure to nitrosamine while maintaining a high-quality drug product that is safe and effective for its indicated use for the American public. We are using surrogate compounds for assessment, but they come with their own limitations. Of those surrogates, it is important to identify compounds with robust carci data. We do apply chemical informatics approaches to inform potency. We look at structural similarity, metabolic activation. Some of these nitroso impurities are bulky, and considerations of

how size and steric hindrance impacts their potency is another challenge.

Next slide, please. So, as we head into the workshop today, we have to look back and say over the past 2-1/2 years, we have certainly learned a lot; however, I have described some of the challenges that are still ongoing. FDA researchers play a key role in optimizing testing conditions for nitrosamine safety assessments. We have researchers who are working on nonclinical safety assessments to best characterize mutagenicity and carcinogenic risk of some of these nitrosamine compounds. In particular, it is important to develop a testing paradigm for those that have little to no published data for those impurities that are previously unidentified or uncharacterized. What are some of the key pieces that are necessary to identify an acceptable intake? Bridging this gap in information also requires collaboration with experts in academia and industry, along with our international regulatory partners to identify harmonized risk assessment strategies. This concludes my presentation.

Next slide, please. I look forward to the discussion over the next two days amongst our panel of experts. I want to thank you for your attention, and I would like to acknowledge the colleagues on the Safety Team, Drs. Dorsam, Atrakchi, McGovern, and Karen Davis Bruno. And also the members of the CTECS Nitrosamine Task Force, various colleagues from CTECS, our OPQ colleagues, Office of Generic Drugs and New Drugs, Drug Shortage Staff, Office of Communication and Compliance. We have all worked very closely together over the last 2-1/2 years. So, thank you for your attention, and I look forward to an exciting workshop. Thank you.

DR. ATRAKCHI: Thank you, Dr. Eisenbrand and Dr. King for the comprehensive and information presentations. We now begin with the questions. They are organized under two headings, exposure and risk assessment and chemistry. They focus on important issues and the challenges of impurities in medicines in general and nitrosamines in particular. We are also interested as you have heard in the panel's thoughts on the research needed to

further our understanding of nitrosamines. We begin with the first question under the heading of exposure and risk assessment.

What are the endogenous levels of nitrosamine formation in humans and rodents? Once formed, what is the rate or kinetics of elimination? What are the conversion rates in the liver, circulation levels in the blood, and normal variations? If this information is not available, can it be determined experimentally?

As we know and we have heard from Dr. Eisenbrand, nitrosamines are present in the environment. We also know, not only are we exposed to them exogenously, but they are also formed endogenously. Therefore, it is imperative that we understand their pharmacokinetics in order to determine exposure and ultimately calculate risk. This question asked how much do we know about nitrosamine absorption, their distribution, how quickly they are metabolized, and how fast they are excreted. I would like to start by asking Dr. Hecht to begin the discussion.

DR. HECHT: Yes, so, we know quite a bit about endogenous formation based on studies that have been carried out with nitrosoproline where subjects have been dosed with proline plus nitrite or even proline plus nitrate, and then nitrosoproline can be quantified in the urine because nitrosoproline is not metabolized. It is also not carcinogenic. So, many studies on nitrosoproline formation have been carried out, which demonstrate the endogenous formation of nitrosamine. So, the overall yield is actually quite low based on the amounts of proline and nitrate that are given. But we do not have reliable data for compounds such as dimethylnitrosamine because dimethylnitrosamine is rapidly metabolized in the liver, and we do not have good data on the quantitative formation and excretion of its metabolites. One can visualize why this could be addressed, but it is very challenging. So, while, from a structural activity point of view, you would expect some endogenous formation of dimethylnitrosamine from dimethylamine, for example, in the diet and nitrate and nitrite that are normally

taken in. But quantitatively, we do not have good data because of its rapid metabolism. So, it is still a challenge to determine whether the endogenous formation of carcinogenic nitrosamine, such as dimethylnitrosamine would be far greater, for example, than the exposure from pharmaceuticals. That is my answer.

DR. ATRAKCHI: Next we go to Dr. Kyrtopoulos.

DR. KYRTOPOULOS: Thank you. And generally I agree with what Dr. Hecht has just said. And he mentions that Dr. Eisenbrand that basically three approaches have been used to try and estimate, basically guess, and the formation for dimethylnitrosamine. This is really the only carcinogenic nitrosamine about which we can try to guess regarding its endogenous formation. Based on the concentrations of NDMA that have been found in blood or in urine and some estimates of the toxicokinetics of NDMA and having in mind that it is a very small fraction of NDMA that is actually excreted in the urine, people have tried to come up, and they

have come up with estimates as we have previously of hundreds to thousands of micrograms of total throughput of NDMA through endogenous formation. I can say a little bit more about the third approach towards the same question. Based on the fact that NDMA methylates DNA, it gives rise to methylated adducts, and it is possible to measure methylated DNA adducts in human patients. Of course, there is a question of what is the source of these adducts, but assuming that NDMA is a major source, one can try to use animal data and extrapolate back to how much exposure would be required to give rise to the adducts we know. So, I would like to just take you through this argument. Data on methylated DNA adducts in humans are really quite limited, and most of them have been based on small pilot studies. However, there is a series of studies that we had carried out some years ago, which were relatively extensive. We have measured 0-6 methylguanine, which is an important DNA adduct, premutagenic and precarcinogenic, this one by NDMA, and we had measured it in human blood DNA, that is in blood leukocytes. And I would like to show you

some numbers because I think it is important to have an idea of the scale of what we find.

If I could have the next slide, please. In three studies all together, which we carried out over a period of 10 years or so, we looked at about a thousand blood samples from women citing general environmental exposure. In about 700 of those samples, we could measure 0-6 methylguanine, and we had an average content of 16 attomoles per microgram DNA. An attomole is 10 to the -18 moles. With a range of 4.5 to 109. Sixteen attomoles corresponds to 27 moles per 10 to the 8th moles of guanine or about 59 or 60 molecules per diploid cell. I would like to explain why I use these units of content per cell. This is because repair of adducts is an issue that comes up frequently in discussing response and risk assessment. And it may come up during the discussion of subsequent questions. 0-6 methylguanine is repaired by a protein, a methyltransferase, known as MGMT which acts stoichiometrically for every molecule of adduct that it repairs, a molecule of alkytransferase is destroyed. That means that if

there are enough adducts in the cell to be repaired, and if MGMT does get depleted, that means that the dose response curve might show an upward turn. So, expressing the adducts on the basis of cellular content allows us to compare them with the content of MGMT in differing cells. Now, in experimental animals treated with low oral doses of dimethylnitrosamine (NDMA), blood DNA accumulates more adducts than almost all other tissues expect for the liver, which accumulates a little bit more adducts. So, if the adducts that we measure do come from NDMA, these levels are unlikely to be exceeded by other tissues. In other words, what we measure in blood represents the higher level of adducts in any tissue. As far as I am aware, MGMT content of primary human tissues is substantially some orders of magnitude higher than the highest adduct levels we have seen in human blood DNA. In other words, the 109 attomoles per microgram DNA is much, much lower from 10 to 100 hundred times than the levels of MGMT that are usually found in human tissues. So, that means that it is unlikely that loss of repair is likely to play a role in environmentally

relevant exposure levels.

Now, how likely is it that these others come from NDMA. We know about a dozen methylating agents -- experimental, industrial, medicinal, of endogenous origin to which humans may be exposed. From studies in rodents, we know that NDMA is by far the most efficient chemical capable of giving rise to 06-methylguanine in blood leukocytes in-vivo. Therefore, taking into account the degree of human exposure to these chemicals, I think it is not unreasonable to think that NDMA is probably the most likely source of these adducts in the human tissues that we measure. So, assuming that this is so, we can attempt to estimate the exposure that is required to give rise to the others that we see based on those responses in animals. There have been many stoichiometric studies published at times. Many of the older studies have used quite high doses of methylating agents, NDMA in particular, which likely decreased from MGMT. We have carried out studies using much lower levels of NDMA, non-MGMT depleting, and so we have those response information, primarily

in rodents buy also in monkeys, in patas monkeys. What we have found that if the dose of NDMA is expressed not as an amount per kilogram for the weight but as amount per square meter surface area, and if those response curves for adduct accumulation in blood DNA in different species become quite compatible, well within -- the slopes are within a factor of 5 easily. And I should add that we have also found that the rate of repair of 06-methylguanine in blood leukocytes is similar in rats, in monkeys, and also in humans who have been treated with methylating drugs. So, if we can go to the exposure response curves.

Can we see the next slide, please. On the left, you can see the adduct accumulation curves in blood DNA of rats treated chronically with NDMA in the drinking water, and on the right, you see the dose response curve for the steady state levels, which is fairly linear. The dashed horizontal line corresponds to the upper limits of adducts measured in humans, and from that, we can see that the corresponding exposure is just under 500 micrograms per square meter, which adjusting, extrapolating to the human exposure would

correspond to 982 micrograms per day. That is for the maximal adduct levels, and for the mean adduct levels, something around 144 micrograms. So, we are speaking again about background NDMA exposures of hundreds of micrograms per day, which are much higher than those that are derived from external exposures. And therefore, they are likely to be of endogenous origin. And, of course, these numbers are in the same ballpark as those presented earlier by Dr. Eisenbrand, coming from the Hrudley publication of 2013. For some reason, they had used our data to come up with other higher numbers. In any case, this is where we stand. Just two words about the uncertainties of this analysis. First, I liked Dr. Eisenbrand's statement about the need to validate the measurements. And we used immunochemical methodologies, and the immunochemical methodologies when pushed at their limit of sensitivity always had question marks. We had taken steps to minimize these question marks, but one would like to see measurements of DNA adducts carried out by more reliable modern methodologies, analytical methodologies. Secondly, we are not really

sure about the similarity of the dose response curves in humans with those of the rats that we have used. And perhaps an important thing to also have in mind is that we really do not understand the determinants of the endogenous formation of NDMA. No known studies have really been carried out to answer this question.

And I stop there. Thank you.

DR. ATRAKCHI: Thank you. Now, we move to Dr. Rice.

DR. RICE: First, I want to thank Drs. Eisenbrand, Hecht, and Kyrtopoulos for their comprehensive overviews of some of the issues associated with endogenous levels of NDMA and other nitrosamine formation. And I cannot add a great deal to what they have already presented. I should just like to draw attention to the fact that NDMA and other nitrosamines largely are metabolized by P450-2B1, and that metabolism is subject to competitive inhibition by simultaneous administration of other substrates for that enzyme. Dr. Kyrtopoulos especially has done a lot of work with ethanol consumption concomitantly with the administration of nitrosamine in experimental

animal and better suited perhaps, so I can raise this issue. But the basic point is that the distribution, excretion, and so forth is not something that is unchanging but is very dependent on what other exposures are simultaneously occurring. But great shifts both in organ distribution of methylating effects, as well as excretion of nitrosamines can occur when substances compete for p450 simultaneously in my experience. Consequently, I would just note the need to keep in mind in efforts to understand the levels and adducts of elimination such that it is very much dependent on what else is present in an individual. Thank you.

DR. ATRAKCHI: Thank you. Dr. Eisenbrand. I know you have spoken quite a bit, but maybe you can add a little bit more.

MR. EISENBRAND: No, I would like to actually. Thank you very much. I think this Question 1 is one of the most important questions of the whole meeting here because I think we really need to get reliable information about endogenous exposure, especially to dimethylnitrosamine but not exclusively.

There are other compounds as well as Dr. Shukars [ph], and some have shown a couple of years ago. Methylation is also carboxymethylation and some others. So, as I proposed at the end of my talk, we really need to revisit this endogenous formation and exposure question quite a bit. I think it is very important also to put in relation what happens by potential exposure to drug constituents. And it is not only that, but the second point is also as I mentioned in my talk as well that we also need an updated database on exposure from food to compare with potential exposure from drugs. I mean, if you look into the proposed AI levels, the acceptable intake levels, these are maybe from the TD50 values. Some of them have a good database and data density but most of them not. And in my opinion, we end up with a series of theoretical values in the nanogram range, which is all right, of course, as a safety measure, but we need also to have this view of what happens in real life, and that is my meaning as a toxicologist. We need to know what we are normally exposed to, not only exogenously maybe by our nutrition but also by the

endogenous exposure. Nowadays, we have the means to measure that. It is not the situation of 30 years or 50 years ago. And as Dr. Kyrtopoulos pointed out, I think one biomarker that is already very well usable, that is 06-methylguanine. And again, we have to have the adequate PBPK random models and come to numbers that are really dependable. I think it is very important.

DR. ATRAKCHI: Thank you. And Dr. Bucher.

DR. BUCHER: Yes. I agree that the improved methodologies have improved data for endogenously generated nitrosamines and are very good and very useful in this context. I am somewhat afraid that it is going to take quite a while to generate this information and to be able to actually contribute to this discussion. I think it is going to be perhaps necessary to make some other considerations that I think will be coming out in the discussions to some of the other questions later on. So, I would hope that we would keep experimental work going in this area or restart experimental work in this area. I think there

are some practical issues related to the timing of generating data to answer the questions that are really, really on the table at the moment. Thank you.

DR. ATRAKCHI: Thank you. And Dr. Zeiger.

DR. ZEIGER: Thank you. I think the earlier speakers have pretty much covered all the points pretty well. One additional item I would like to interject is whenever we do these types of studies, we assume that the kinetics and the potency of the mutagenic or carcinogenic response will be similar in humans and in the test rodents. And we know at least from mutagenicity studies that there is quite a bit of difference in the rate of activation by liver of these various nitrosamines and differs quite widely just between mice and rats and hamsters where we have data. We have no idea how those measurements would translate to a human exposure in a human situation. I just wanted to raise that point now. But other than that, I have nothing to add.

DR. ATRAKCHI: Thank you. Very good points. And Dr. Adamson.

DR. ADAMSON: I think the talks by Dr. Eisenbrand and Dr. King were very helpful, but I think the additional comments that Dr. Eisenbrand made are particularly important because the endogenous formation from both the data and the literature and what Dr. Kyrtopoulos presented shows magnitudes of formation of endogenous nitrosamine, particularly DMNA much higher than we are getting in the medicines in which it has been detected. So, I think with the newer techniques, analytical techniques need to be applied to both endogenous formation and particularly also with food. Because the amount in food varies from country to country with the current analytical techniques and also varies between various investigators. So, I think using the new analytical techniques should help us because at the present time, the endogenous formation of nitrosamines and the amount in food overwhelms what has been found in the medicines to date. So, I would emphasize that what Dr. Eisenbrand said, we need to go back and look at food and endogenous formation with the newer analytical techniques. Thank you.

DR. ATRAKCHI: Thank you. And Dr. DiNovi.

DR. DINOVI: I don't actually have much to add, but based on what we just heard there, I would agree. It does appear as though the endogenous exposure is going to overwhelm the food. There are differences from country to country, but it is comforting that if you look at the surveys that have been done -- and I'll talk a little bit about this this afternoon probably -- they are relatively similarly, and the role in that sub 1 microgram a day range, endogenous will have to be considered further. Thank you.

DR. ATRAKCHI: Thank you. I think we will discuss this a little bit later on with the other questions. But one issue that to me seems very important is we need to have analytical methods that can distinguish between endogenous and exogenous formation. Otherwise, the data will not be very accurate. With that, I would like to move on to the second question.

Can nitrosamines be classified? If

yes, what is the basis of their classification? Could they be classified based on carcinogenic potency, on their chemical structure, on the chemical reactivity, direct alkylating agents versus those that require metabolism, or based on the adducts that are formed as just heard, the 06 or the N7 methylation? Any other basis for classification? And once we choose a classification, what is the basis of using that over the other ones? If classification is not possible, is it feasible to calculate a single, acceptable intake value for nitrosamines? That is we can come up with a class-specific limit using the existing carcinogenicity study results of over 100 nitrosamines irrespective of the study quality. It seems that the main concerns for pharmaceuticals and maybe biopharmaceuticals are the volatile nitrosamines in particular. As noted earlier, since the discovery of the toxicities of these nitrosamines, much of the carcinogen assessment studies were done in the '70s through the '80s and early '90s by scientists here in the U.S., as well as abroad, some of whom are as we already noted are here with us today on the expert

panels. Nitrosamines were shown to be toxic, both in androgens and carcinogens. They are also teratogens. They have a wide range of potency on order of magnitudes, and the majority cause cancer in around 40 animal species. They cause tumors in multiple organs, different durations of exposure, some would induce tumors after a single dose even though their half-life is short within a few hours.

They also have different latency. All of this makes classification of nitrosamines that are quite a bit difficult. However, it is an important and critical aspect of what we are trying to discuss today at this workshop. And with that, I'd like to start with Dr. Eisenbrand.

DR. EISENBRAND: Thank you, Dr. Atrakchi. Again, a very important question is going to the classification of nitroso compounds. Of course, one can use a classification based on trying to develop a system for carcinogenic potency and rating, and of course, that has been carried out before already, based mainly on the TD50 values that are in the former Gold database and the CPDB. And it

is a way of doing that. Probably at the moment, it may be the best way to go on until further questions have been addressed sufficiently such as the ones we have just discussed before. It is the relation between the drug-mediated exposure to the exposure that is coming from food unavoidable or from endogenous exposure. And I agree that this may need some time to systematically do the research that we can depend on and for that time being, it may be the best way to go just with the deferred proposals of group-specific values concerning the acceptable intakes based on the TD50 procedure. So, this is a way to go. And the second point, of course, it is a complex task, but it can be simplified because we know already about defined chemical structures that inhibit carcinogenesis or mutagenesis. As we have heard, these are the tertiary butyl groups, and these are the ionic compounds like the nitrosated amino acids. And on the other side, there might be also protonation that is important to reduce bioavailability. So, there are possibilities to look into this with more defined questions to answer.

The chemical reactivity of course that would be the first thing to consider, that nitrosoureas for instance or carbamates or this other compounds that do not require metabolic activation, they react by themselves, and this is a consideration that is also important when you consider stability questions. For instance, I would personally think that nitrosureas are not really very stable, so it might be that there comes stability issues into consideration showing that within a certain time of let us say storage of so, these compounds may be done. Of course, this is open to research. It has to be really looked into quite closely. But just as a potential point of view. And what I would personally think is very promising is to use the biomarkers of epilation. 06 methylation or carboxymethylation or some others, I think that is a good way to go, and it should be really substantiated by I would say PBBK modeling of the enzyme activity that is going on and formation and repair and all these things. I think we are much better today to address these questions in a reliable way. So, that could also be a good

possibility.

In terms of other I do not have very much further to add, and the classification I may just recall you that EFSA (European Food Safety Agency) has proposed a couple of years ago the margin of exposure methodology where you use a benchmark dose as obtained in animals, mostly on the low side of the dose response score, at least not more than 10 percent population percentage of the dose effect or even lower. And do that with the appropriate modern methods of modeling. And then if you have this BNDL value or BNDL 10 or BNDL 5 or around that area or even lower, then to use for risk assessment the distance to human exposure. And of course, again, we come back to human exposure, but this human exposure I think has considered overall exposure, the real-life exposure. And then determine the margin of exposure between this BNDL value and the exposure of the consumer nowadays on average. That I think I would think is a good way to good. EFSA has tentatively said that if this margin is more than 10,000, the space between the BNDL value and the consumer's exposure, then one could say

that is of very low concern, no primary concern. And I think it is a way of addressing these things. My idea would be that this is also a good way to go. So, I stop here. Thank you very much.

MS. ATRAKCHI: Thank you. Dr. Bucher.

DR. BUCHER: So, to me the key to this question is can nitrosamines be classified. Whether they should be classified or not, under certain conditions, that is another discussion. But to the question of can they be classified, I would agree that there is a qualifying yes. Chemical-structured-based models have been published, and do a reasonable job of classifying nitrosamines as carcinogenic or not. A few models have attempted to classify nitrosamines according to their carcinogenic potency using the TD50 values as described earlier. And while I agree that the benchmark dose calculations and the margin of exposure models are better than the TD50s, given what we have to work with, I think the TD50s are going to have to remain as part of our considerations. Nitrosamines have also been classified by quantitative structure activity, relationships using structural

alerts for carcinogenicity with some success, and of course, expert judgement and additional experimentation is also valuable and needed and will need to be used along with modeled results to improve the predictivity of these models. Many of the nitrosamines that have been identified as contaminants in drugs require metabolic activation, so such things as susceptibility to P450 hydroxylation in the alpha carbon. And also important is the half-life of the resulting diazonium ion and reactivity of the carbynium ion. All of these things are very important to consider with respect to expert judgement, and including in new models. To widely use these parameters, they would need to be predicted in many cases, so this would reduce the confidence of the outcomes of some of the models. So, to me, there are a variety of classification modeling approaches that could be and have been applied to this question. But so far, even the best of the models are only pretty good. They are very far from perfect, and some are fairly computationally intensive. As to which model, I would choose for FDA going forward in the absence of

anything better, I would choose one of the reasonably predictive QSAR models and incorporate carcinogenic potency using the TD50 values and measured with a heavy dose of expert judgment.

Turning to the question of the feasibility of a single acceptable intake, I believe this is possible. Again, and relying on the carcinogenic potency database. If one simply scans the estimated TD50 values for the over 100-plus rodent carcinogens for nitrosamines in the database, they can reasonably be placed within some ranges. In a few potent rodent carcinogens with a lifetime daily TD50 doses below 1 mg/kg, many of these have values between 1 and 10 mg/kg per day and others between 10 and 100 or even higher. Those with a very high TD50s can probably be ignored insofar as human hazards from drug contamination is concerned, and acceptable intakes could be calculated for substances falling within these high potency ranges. And it seems reasonable to use the European Medicine Agency's proposed linear dose extrapolation based on either the most potent or the median nitrosamine potency in the range to the

risk level of 1 in a 100,000 as proposed in the AMA report. Actual TD50 values or modeled estimates could also be used, but you should recall, of course, as pointed out earlier, the potency estimates from the rodent cancer studies are very imprecise. They depend on a whole list of factors having to do with the study design and the power to detect increases in tumors, to study at length of the extent of histopathologic evaluation in these studies, and other factors related to the way the study was performed. With that said, I think the data probably have value in predicting relative carcinogenic potency and perhaps if used within these various ranges of TD50s that I've mentioned, they may be useful, and clearly, they have already been incorporated into some of the existing published models of nitrosamine carcinogenicity. I think I'll stop there.

DR. ATRAKCHI: Thank you. And Dr. Guttenplan.

DR. GUTTENPLAN: I don't have too much to add. I just have the feeling that we need to have some way of superimposing the difference between human

metabolic activation of carcinogens and the rodent data. I am not sure the best way to do that but maybe with some model compounds, at least, it would be possible from what we know about human data to compare it to rodent data. And maybe there is some way of adjusting the carcinogenicity from rodent values into human values, possibly by looking at their ability to form say 06-methylguanine if you have a carcinogen in rodents that is very good at forming it, and it is not so good in humans it would suggest that there are metabolic differences or pharmacological differences that might account for these differences. So, I would say the carcinogenic potency is the first stage, and it is probably the best we have at the moment. But I would suggest that there are improvements that could be made. And that is about all I have to say on the issue.

DR. ATRAKCHI: Thank you. Dr. Zeiger.

DR. ZEIGER: Right now what we use is primarily mutagenicity versus non-mutagenicity, which is at first the Ames test, which is the first test generally applied to these chemicals. And obviously,

the ones that are mutagenic are presumed to be carcinogenic. You know, unfortunately, as I have mentioned, I think I mentioned before, the mutagenic potency does not correspond to the potential carcinogenic potency with these chemicals. We have in the Ames test a mutagenic potency range of about four or five orders of magnitude, but whether these compare with carcinogenic potency, they generally do not. We heard before that, for example, nitrosodiethylamine has a higher carcinogenic potency than the diethyl form. But in the mutagenicity studies, they have equivalent potencies. So, that does not help. I think what we really need, and it has been addressed before, is that we need to have more information on the human metabolism of these substances. We do not have very much on in-vitro human metabolism using either metabolic incompetent cells or just liver homogenates. Without this information to compare it to the rodent information, I am not sure if we can go much further than going through just like basing it on structure, basing it on DNA alkylation. Whether it is possible to calculate a single acceptable intake, I do

not think we have enough information to even address that question at this point. And that is all I have to say at this point.

DR. ATRAKCHI: Dr. Adamson.

DR. ADAMSON: I think the response that Dr. Guttenplan made I would echo, that we have to remember this is a TD50 or benchmark dose based on rodent data and that the human data both with regards to activation of the carcinogen and the alkylation may be different. So, we have to keep that in mind. But at the present time, I would agree the best we can do is either use the TD50 or benchmark dose in rats. But I think further work needs to be done to try to relate this to humans.

DR. ATRAKCHI: Dr. Cronin.

DR. CRONIN: Yeah, thank you. I firmly agree with all of the previous comments, and when considering classification, we need something to base the classification on. We have the TD50. We also have the possibility of going to BNDL. I think, as well, from my perspective, it would be interesting to review the data and see if we are just going to

classify as carcinogenic-noncarcinogenic, that is one issue. If we want to look for potency classifications within the data, then we need to look at the data, and I am aware, for instance, from Professor Eisenbrand's presentation, we talk about high potency. We talk about low potency. And there have been some efforts to quantify that a little bit more. So, can we investigate the data to see if there are natural fallouts in terms of TD50 or the BNDLs in terms of potency. I also take on board all of the comments about reactivity and metabolism. I am intrigued to know is there a direct correlation between reactivity and carcinogenic potency. I suspect not because of all of the other issues that are involved in it. That is something again we need to tease out. Can we measure reactivity itself? We have done in other instances. For instance, for protein binding, or probably we have less data for reactivity for DNA binding. And I am intrigued by the suggestion. I had not really thought it before, but I think it is an excellent suggest to consider biomarkers, measures of reactivity, particularly if we can extrapolate up or

we can use human data.

I would also like just to think -- obviously we need short-term and FDA needs short-term achievable goals. A lot of those have been articulated but also to think where we are going in the future with aspects such as classification. I would just like to raise the issues. For instance, it can be given by Bayesian modeling probabilistic-type modeling of how we can incorporate data, how we can incorporate knowledge in different lines of evidence. So, that could be structural activity relationships. That could be biomarker data or metabolism-type data. And the reason I raise this is because it does give us the possibility of being able to assign some kind of level of probability and certainty to prediction. And I am very taken by the thoughts in the moment of rather than thinking of a TD50 as a single value, it is a distribution, and what we are trying to do is narrow that down to make a decision. Such as, for instance, to be able to find acceptable intake values.

With regard to acceptable intake values, I do not have any more specific comments. It

does seem a little bit analogous to TTC. I know that is probably a strange thing to say as we have already identified the nitrosamines as the cohort of concern, which is automatically removed from TTC, but maybe there are other ways or more data or more knowledge we can take from the TTC paradigm. And just to mention, there will be a workshop in Europe in the next few weeks on carcinogenicity and updating the TTC paradigm for that. Other than that, I do not have any more comments. Thank you.

DR. ATRAKCHI: Thank you. And Dr. Kyrtopoulos.

DR. KYRTOPOULOS: Thank you. I think it has all been said actually. The only thing I would add is that because a number of people refer to this issue of using biomarkers, in other words the DNA adducts generated by the nitrosamines. The extent to which they could serve as markers for potency or markers of risk and so on. The trouble is that despite all of the work that is being done on the nitrosamines, I do not think that we really understand the mechanism by which all the carcinogenesis in

sufficient detail. For the simple ones like dimethylnitrosamine, the other methylating nitroso compounds, methylnitrosourea, and so on. Okay, 06-methylguanine seems to be potent in various animal models. But the minute you go to more complex structures, and especially with regard to the chemicals out of concern in relation to the current issue, drug contamination, where the structures are quite varied, and I do not think that some of them are quantitated, and certainly the cyclic nitrosamines and so on, we do not really know whether it is 06-alkylation or whatever other adducts are. So, I am not really very optimistic that they would be, based on what we know today, a very practical guide toward helping us to classify. I guess if a chemical is giving rise to 06-methylguanine, yes, it would be likely to be a more potent carcinogen, but that does not tell us much about many of the other chemicals. So, I would eventually fall back to animal carcinogenicity combined with some expert judgment in relation to chemical metabolism, conversion to alkylating agents, and so on along the lines, which

have been presented previously. That is it.

DR. ATRAKCHI: Thank you very much. We are close to a break of 10 minutes, but before we go, I would like to ask all the other panelists if anyone has anything to add for the first two questions that we went through right now. Please go ahead if you would like to comment.

DR. HECHT: Yes. This is Steve Hecht. I think we need better measurements. I think there are way now to look at DNA adduct formation in humans, and I think we need to do that more thoroughly, more precisely, more reliably using the currently available high-resolution mass spectrometric methods to really determine how much relevant DNA damage comes from nitrosamine formation and nitrosamine exposure in humans. So, I do not think we really have that data, and it is quite critical for the risk assessment. Thank you.

DR. ATRAKCHI: Thank you. Anyone else?

UNIDENTIFIED PANELIST: Dr. Kyrtopoulos, you gave some data from the blood levels of 06-methylguanine and sort of extrapolated back to

an intake of dimethylnitrosamine. Is there any way to determine how much of that came from DNN and how much came from other sources?

DR. KYRTOPOULOS: Well, not directly, but as I indicated in the beginning, I am aware of maybe a dozen chemicals, to which potentially there is human exposure and which are capable of methylating DNA. There is s dimethylnitrosamine. We know other chemicals to which humans are not likely to be exposed like methylnitrosourea. There are also chemicals like dimethylsulfate, iodide, and so on. There are medicine drugs such as temozolomide, which all give rise to DNA methylation. We worked with quite a few of those chemicals in experimental animals, in rodents, and it turns out that NDMA really stands out. It is the most efficient generator of 06-methylguanine in blood DNA. I emphasize that I am speaking about blood because blood does not metabolize nitrosamines. So, it gets methylated as it goes through various tissues that generate the intermediate methylating agent. So, keeping in mind how likely people may be exposed to these chemicals that I have named, one does

not have very much left. Endogenously generated amino acids, glycine for instance. Carboxymethylate and methylate, so that is a potential source of endogenous methylation. However, from the data that we have, the methylating ability of that intermediate because it is a stable chemical seems to be quite low. So, taking everything into account, animal data, animal dosimetric data and human exposures, NDMA seems to be the most likely source of this adduct.

UNIDENTIFIED PANELIST: How about NNK?

DR. KYRTOPOULOS: NNK on a per dose administered dose basis it methylates much less than NDMA. Dr. Hecht may have the numbers. I do not have them in my mind right now, but I remember at the time when we worked on it, it could not be compared with NDMA.

DR. HECHT: Yes, that's correct. We compared that -- we published a paper in 1986 on that. NDMA is a better methylating agent, but also NMK.

DR. KYRTOPOULOS: That's right.

DR. ATRAKCHI: Okay, then. Thank you very much. And we will take now a 10-minute break,

and we'll resume at 11:40. Thank you.

MS. PAINTER: All right. It is just one minute after 11:40, so, we are going to begin with the next question.

Just as a reminder for everybody, please utilize the Q&A box, to submit your questions. While we do have a team of people moderating the questions, please know that we will not send a response. However, if you see that your question is dismissed, that means that it has been received and sent to the moderators. Thank you.

DR. ATRAKCHI: Thank you. We move on now the third question.

The carcinogenic potential of nitrosamines is dose and duration dependent. Is there an in-vivo exposure level for nitrosamines that could define low versus high risk for carcinogenicity? Is it appropriate to calculate a now-observed-effective-level dose for carcinogenicity? What are the criteria to do so? Would a resultant in an Ames negative be adequate, in vivo mutation assay negative, or another other test?

The second part of the question is can a less than lifetime approach as described in M7 Guidance be used to determine the acceptable intake of nitrosamine if the drug is indicated for a short period of use?

Based on the discussion so far with the understanding that humans are exposed endogenously and exogenously to nitrosamines, we know some of the pharmacokinetics. We also know that DNA repair capacity varies tremendously among humans as well as among animals. And there is also the less ideal quality of the carcinogenicity studies conducted with nitrosamines. With all of this in mind, can a NOEL be identified with confidence? Some of the studies have shown clear and abrupt transition to a no effect. Other chemicals showed gradual change with a curvilinear dose response and a sigmoidal in the low dose. Another consideration to keep in mind is the dose rate, is the interval between the doses and how would this affect the DNA repair. Earlier studies have show cancer rate is independent on age, and DEA for example when administered at the same dose to

animal species of different life expectancies all animals developed tumors at the same rate and time. Regarding the second part of the question, a less-than-lifetime approach, how reliable are the models that extrapolate from long to short duration. What is the model sensitivity and the shape of the response, nonlinear versus threshold, for example. In the end, can an acceptable cancer risk be achieved based on exposure to a predefined limit for one or more nitrosamines that are known to be potent mutagenic carcinogens when exposed only for a short period of time?

We will start with Dr. Bucher.

DR. BUCHER: Thank you. You laid out a large number of questions there that are quite difficult to respond to, but I will start with the question of whether there is an in-vivo exposure level for nitrosamines that could define low versus high risk for carcinogenicity. I think that one must simply take a practical approach to this question and look at the approach that has been laid out in the European Medicine Agency's report. As an example, a

practical answer would have to be that an in-vivo exposure level of high-risk would simply be the adjusted human dose representing more than a 1:100,000 risk calculated based on the carcinogenic potency database. This would mean that a nitrosamine dose of high risk would be one with a TD50 extrapolated dose of greater than 1.5 mg/kg per day for a nitrosamine with a TD50 less than 1.5 mg/kg per day. By definition then, a human nitrosamine exposure of low risk would be an extrapolated TD50 dose of less than 1.5 mg/kg per day for a nitrosamine with a TD50 greater than 1.5 mg/kg per day.

And with respect to the second question about it, is it appropriate to calculate a NOEL dose for carcinogenicity? I personally do not think that the concept of an experimentally derived NOEL is appropriate for genotoxic carcinogens and generally for genotoxic compounds in general. In the example I just mentioned, the NOEL is in essence the dose defining the risk level low or below 1:100,000 because this is a generally agreed upon acceptable level. A second hypothetical NOEL in the dose where the

additional risk from exposure to an exogenous nitrosamine falls below the risk from nitrosamines that are generated endogenously. In this case, I see two ways of looking at this information. One is that a low NOEL would simply be the exposure dose where the risk presented by the exogenous nitrosamine is below the absolute total risk from endogenously generated nitrosamines. The counterview would ignore the risk from endogenously generated nitrosamines and consider that exogenous nitrosamine exposures would always present an additional incremental risk that can be calculated as in the example I mentioned earlier. I believe the second view is more ethically defendable and that an incremental risk is still a risk. I note that this concept is going to be further discussed and addressed in Question 5, so I will leave it at that for now.

With respect to the less-than-lifetime acceptable daily intake approach as outlined in the ICH M7 Guidance, I understand the concerns, especially those that you mentioned, given the experimental animal cancer data that might lead one to question

whether this is an appropriate practice based on data on the observations of the higher potency of some of these nitrosamines if given in say high dose post dosing rather than in long lifetime lower-level doses. But I am in general agreement that given low doses that correspond to the very low risk levels that we are talking about, the 1:100,000, that to exceed the acceptable lifetime intake levels for shorter periods of time probably does not represent an unreasonable risk for adults and likely for patients starting even at younger ages. But when you consider that some of these models of short-term rodent cancer studies, especially the neonatal mouse model, in particular in their response to short exposures to genotoxic agents showing carcinogenesis, I would suggest that based on these that the risks of the less-than-lifetime approach may be more significant in children, and I think that this whole area needs a whole lot more discussion and careful consideration. Thank you.

DR. ATRAKCHI: Thank you. Dr. Adamson.

DR. ADAMSON: I would agree that use of the dose for additional risk of 1:100,000 would be

appropriate. I would say to use the benchmark dose rather than the TD50 would be appropriate from the rodent data, remembering you are extrapolating from the rodent data. But I would also say that with regards to the second part of the question about a lifetime approach, yes, I think it is appropriate because I believe that the induction of cancers, dose times time plus the repair mechanism, so I think the use of a lifetime approach is fine, but if it is a short duration of use, I think the present application that FDA uses is appropriate to determine the dose.

DR. ATRAKCHI: But are you saying that the use -- you are agreeing to use the less-than-lifetime approach if the medicine is used for a shorter period, not a chronic use, you are agreeing to adjust for that or not?

DR. ADAMSON: Yes, I'm saying that I think you can adjust for the fact that it is less than a lifetime use.

DR. ATRAKCHI: Okay. Dr. Kyrtopoulos.

DR. KYRTOPOULOS: Yes. Well, I am trying to think a little bit in kinesthetic terms. I

would like us to remember the Peto rat mega bioassay that is used to derive what is today considered the acceptable intake. In that study, what they observed a dose response curve for the induction of liver cancers, which was hockey stick-shaped as it was called. We have the slide. On the right, the dashed lines show the dose response for the induction of different types of liver cancer in the Peto bioassay. It was expressed with it by a parameter called the Weibull index. And you can see that at a dose rate of about 200 mcg/kg per day, there is a sharp upward turn. However, below that exposure, the dose response curve was described by Peto as linear with no evidence of a threshold. And this linearity in absence of a threshold ties up with the data that we have on adducts in rat liver, which is a continuous line above. This is the data formation from an animal which basically replicated, repeated the Peto bioassay. And you can see that the other dose response is pretty linear all the way down to very low doses. So, there is no break in the other dose response curve. What happens around 200 mcg/kg per

day is that there is an increase in the induced cell proliferation in the liver, and that is one factor, which contributes to this upward turn of the carcinogenesis exposure response. So, both on the basis of the bio assay and the adducts dose response, the dose response at low dosages is linear, and there is no evidence of a threshold. We should say, of course, that this is what happens in animals, in the rats. We do not know whether the same thing applies to humans, and it is actually something that was already pointed out in the Peto paper. However, based on what we know from the animal data, we do not see any evidence of a no-effect dose.

So, can turn to the second question regarding the application of a less-than-lifetime approach? Carcinogenesis is a function of the accumulated dose, the accumulated carcinogenic damage. But it also depends on additional factors as we see. It may be cell proliferation, maybe other biological phenomenon, apoptosis, and so on. As far as the DNA damage part is concerned, the fact that we have linear dose response means that it is defensible. It is

acceptable to accept an exposure to a higher dose for a shorter period of time because the integrated overall lifetime exposure in terms of DNA damage would remain the same. On the other hand, we do not know what the dose response relationship is with regard to the other factors, which contribute to the carcinogenesis overall. So, we do not know how cell proliferation or the reduction of cofactors and so on may vary if temporarily increase the dose. It is not so easy to say that the effect of a higher dose for a shorter time is equivalent to a lower dose for a longer time. Nevertheless, on the other hand, we do have the real problem of the possibility that we may have to live with the presence of an undesirable chemical in a medicinal product. So, I think the overall evidence that we have here would make it possible -- it would be acceptable for me to accept a higher than the lifetime-acceptable intake limit. However, one should have in mind the unknowns, which are present, and keep this exceedance as low as possible. That's all.

DR. ATRAKCHI: Dr. Zeiger.

DR. ZEIGER: Thank you. My comments are mostly regarding to calculation of a NOEL dose of carcinogenicity. And I've never been a big fan of the NOEL calculation because it is very highly dependent on the test protocols that are used to generate the data. For example, you know, most carcinogenicity, most in-vivo mutagenicity studies are done at subtoxic doses for in-vivo for long-term subtoxic doses, whereas the human exposure is generally on orders of magnitude lower. And we assume that there is a linear extrapolation, but also that extrapolation is based on the dosing and dosing regimen that is used in the studies. I think we already classify chemicals of nitrosamines as Ames test negative and Ames test positive. With Ames test positive being presumed to be carcinogenic unless they are shown otherwise. And the majority of Ames test negative studies to my knowledge are noncarcinogenic. With regard to using in-vivo mutation assays, we do not have that much data on nitrosamines from the in-vivo studies. The in-vivo studies tend to be less sensitive than the in-vitro studies, and they are also conducted at high subtoxic

or up to toxic doses, but the advantage to them is they are done on blood cells, whether red or white blood cells, which have been shown earlier to be good indicators of the maximum DNA damage dosing you are going to get in-vivo. So, the mutagenicity of gene tox studies can be used to at classify the nitrosamines, but with regard to the potency, I am still stuck with the information that I have that the potency at least in the in-vitro studies does not predict the potency in-vivo, and we do not really have much date to determine how well the potency of the in-vivo mutation assays will predict the predict the potency of the cancer assays. The DNA adduct data may be linear, but to go from the DNA adduct to a mutation requires a number of steps, some of which are toxic, some of which will produce a mutation. Then, to go from the mutation to the cancer, you need another number of stages, any one of which could fail and not give you a cancer result. So, the linear extrapolation from an adduct to a mutation is still very tenuous. I think we have seen that the adduct to mutation studies where we have the data tend to be

nonlinear in appearance.

With regard to the less-than-lifetime approach, I think that is an important consideration. And as we have shown in some of the neonatal mouse studies, which are mentioned, a short-term dosage approach in younger animals, in neonates, can give you different results than the same approach in adult animals. So, the less-than-lifetime approach really needs to be investigated a lot more with regard to nitrosamines.

And I think that's it for me.

DR. ATRAKCHI: Thank you. Dr. Eisenbrand.

DR. EISENBRAND: Everything has been said already to that question. I think personally I would not favor very much an NOEL approach. In my opinion, it is better to use the BNDL approach, especially since the BNDL approach takes consideration of the whole dose range with a specific regard to the low-dose range. And that is why I think it appears to me as more stringent than the TD50 based values concerning NOEL. And that is the one thing. The

other thing, the correspondence between Ames positive or negative and carcinogenicity positive and negative, which Zeiger had already alluded to very convincingly. So, to me the less LTL approach, the second question, I think the reservations concerning the LTL approach consider mainly because of the possibility of intervening repair. And I think this question needs to be decided with respect to the expectable dose that is being taken up by the drug as a contamination or by other ways because this fear that the repair, especially the demethylase repair, the 06-demethylating repair may be not really substantial in this very low dose that we are discussing at the moment. So, from this point of view, I would think, yes, one could use the LTL approach at least for a certain time until scientific evidence shows that it is useful or even it is not. In that relation, I would also mention that there has been a very thorough dose response study by the Dulthai [ph] Group many years ago in the '60s where they used DNA in very widely spaced daily dosage, coming from the upper end of about 10 mg/kg down to as low as 70 mcg/kg

bodyweight. And there it is quite interesting to see that the slope of all these dose responses remains very parallel, very similar down to the lowest level, which still within a lifetime I most say, an extended lifetime of three years of the rats still produced tumors. So, the overall cumulative dose diminished in response to the lower daily dose quite significantly, which shows that even at the very low dose end and of 70 mcg/kg bodyweight per day, there is a sort of -- without any losses actually of the carcinogenic activity. Let's say the DNA mutations used by diethylnitrosamine, there is still a clear dose response seen. So, they calculated from this dose response, the time dose with an exponent of 2.3, so it is a very important parameter to consider that the time of these nitroso compounds goes in a relationship. And so, maybe that the LTL approach really is defendable when we are in the very low dose range. The dose range should be connected to induction of tumors, at least as animal experiments teach us.

DR. ATRAKCHI: So, to your point

towards the end, do we know with reasonable accuracy that the DNA repair mechanisms in enzymes do not get saturated, animals or humans, when we give a large dose of a nitrosamine? And that is not necessarily -- we have to clearly understand whether this nitrosamine is coming from the medicine, not only the medicine alone, but obviously we need to take into consideration the collected totality of all sources of nitrosamines that are taken in. We do say it is dose and duration dependent, so going to taking a big dose of nitrosamine, are we saying that is okay to adjust because the DNA repair mechanism is capable of that. And so, I have another question, but can anyone comment on this?

Perhaps to address your question directly, the question always is what is a big dose. I would not think that in the dose response relationship the upper dose range would be useful to consider. I think that this exercise in risk assessment beyond the lower dosage definitely. If not on a very low dosage, and there I think it is probably not really of great relevance that we have to consider

saturation effects of this demethylase. As soon as you go into the higher dosage, then certainly you have this quite clear.

DR. ATRAKCHI: Okay.

DR. ZEIGER: I'd like to address this.

DR. ATRAKCHI: Yes, please.

DR. ZEIGER: Remember, there are some studies many, many years ago with regard to you talking about DNA repair enzymes but also metabolic enzyme, whether they are saturable. I recall some old VMN studies where once you get above a certain dose of VMN -- I don't remember if it was rat or mouse -- that you start getting kidney tumors in addition to the liver tumors because you are saturating the liver metabolic capability. With regard to DNA repair enzymes, there are two different categories. There are those that are constitutive, which means you always have a certain level of that repair enzyme available in the cell to address the damage, but you also have the inducible enzymes, which means you have to get to a certain level of DNA damage before that enzyme is induced. So, you can get saturation in a

way. You can get saturation of the constituent enzyme, and presumably, you should also be able to get saturation of the inducible enzyme at high enough levels of damage.

DR. EISENBRAND: I totally agree with that. I mean, that's quite clear that you can get separation, especially the experiments where we receive the kidney tumors that happens at high dosages, sometimes even at one single dose. You do not see liver, but you see kidney tumors. But as I said, I think we need to mainly concentrate on the low-dose range because I would not think or I would not expect that enzyme saturation plays any role with the dosage where we are here.

DR. ATRAKCHI: And this is even taking into consideration the exposure from the number of sources that we have already discussed from the environment, from the food. The people's habits. Some of them will eat a lot of smoked foods, smoked fish, and so on. We are not only addressing the level of nitrosamines in the drugs. We know these are comparable to other sources that could be lower. You

all are taking that into consideration, the multiple sources, is that correct?

DR. EISENBRAND: Well, by and large, my impression is that from the multiple sources we have to consider, it is mainly the food because water is so low that in that sense I do not think of any relevance. And the exposure from food still is very, very low. I mean it is in the low microgram a day range, which is nanogram/kilogram body weight. So, I would not expect that it is the nitroso compounds themselves, if there is any influence on enzymes that activate or deactivate in terms of saturation, as well as in terms of repair adducts.

DR. ATRAKCHI: Okay. One more question. It seems like at least based so far on the discussion that potentially the less-than-lifetime approach could be applicable to the nitrosamines that we are addressing here. If that is the case, would safety factors need to be considered and incorporated based on age? And I say this because -- first of all, M7 does not ask and does not require additional safety factors. That is one issue is that it is not -- we do

not use safety factors in M7. Also, our colleagues in the Center for Foods in FDA do not adjust for longer lifespans, and they use models that they estimate now much specific food ingredients is consumed based on surveys of dietary habits. For example, they can limit the analysis to people who consume cheese on a daily basis. Nevertheless, EPA does not assess for carcinogenic impurities in fruits, but they do use safety factors to determine limits in pesticides that are used on foods. So, would you recommend adjusting for less-than-lifetime between let us say pediatric indications versus adults -- medicines used for adults? This would be somewhat not under the guidance of M7, but would you recommend that based on the nitrosamine as carcinogens.

DR. BUCHER: This is John Bucher. This is a very difficult question, of course, and I think that one might pay attention to any kind of information that is available concerning the development of aspects of various repair enzymes according to age. Pay attention to the P450 profile changes with respect to the developing individual.

Certainly, we know they change probably in life. Conjugation reactions change, so all of these things tend to contribute I think to a higher sensitivity of early life stages to carcinogenic exposures. So, I think that unless one takes into consideration the profile of all of these activities, it is really hard to decide whether you need to particularly adjust by a certain factor. That would be my response.

DR. ATRAKCHI: Thank you. And Dr. Kyrtopoulos, would you like to comment?

DR. KYRTOPOULOS: I'd like to say something about the previous discussion on this question of DNA repair and how it may be affected if one is exposed temporarily to higher doses. I really do not think that with the kinds of exposures that we are speaking about even though once potentially coming from the contaminated drugs, there is any likelihood of any significant exhaustion or any significant depletion of the alpha transferase. If you just look at the diagrams from the Peto study where the exposure rates that were used, they go up to quite large doses. In the animal experiment that we did, there was no

depletion, no change in the alpha transferase in the MGMT levels throughout this range. And if you think about the adduct levels that are likely to be generated following an intake of a contaminated drug containing NDMA, I think that the adduct levels that are likely generated, it is concerning. But I think it is very unlikely that they would significantly impact on the pull of the repair enzyme. So, I would not count this factor as one of the items to consider in trying to decide whether an LTL approach is applicable or not.

DR. ATRAKCHI: Thank you. I'd like to ask Dr. Rice to make comments on this question, on Question 3.

DR. RICE: (No response.)

DR. ATRAKCHI: Would anyone else like to comment? We'll get back to Dr. Rice in a moment.

DR. ZEIGER: Yeah. I have a comment. We have been considering thresholds mainly with response to data on liver carcinogenesis, and that it is very unlikely it gets saturated at doses that would be achieved just by intake of contaminated drugs. How

about other organs, though? What is known about the capacity, the 06-methyltransferase repair in other organs? Does anybody know?

DR. KYRTOPOULOS: May I come in? May I respond?

DR. ATRAKCHI: Of course. Certainly.

DR. KYRTOPOULOS: The alpha transferase has been measured in a number of human tissues. As far as primary human tissues are concerned, they all contain quite significant levels. The liver usually has the highest level, but the lowest levels that have been measured maybe let us say five times less. Even in tissues with relatively low levels of alpha transferase, this is orders of magnitude higher than the level of others that we are likely to see coming from all of the environmental exposures and the contaminated drugs. Of course, there is always the possibility that there may be small subpopulations of cells, which are even more repair deficient. But again I emphasize as far as primary human cells are concerned, I am not aware of any deficiency. Cancer cells? Yes. There are cancer cells where the

expression of the MGMT has been lost but not in primary -- I am not aware of data in primary cells showing such an effect.

DR. ADAMSON: Thank you. I would like to make a comment on this question.

DR. ATRAKCHI: Of course.

DR. ADAMSON: We did an experiment in nonhuman primates primarily Cynomolgus monkeys to do a dose response to administration of diethylnitrosamine, and we used at least 10 animals per dose starting at six months of age. And what we found with the diethylnitrosamine giving doses of 40, 20, 10, 5, 1, and 0.1 mg/kg once per week. A clear dose response occurred. At the lower doses, we did not get any tumors at all as long as we did the experiment and when the monkeys were sacrificed, nor did we determine any lesions in the liver or in the organs. That was about 16 years after dosing. The lifetime of cynomolgus monkey is about 20 years. At the lowest dose, only 10 animals per group. At the highest dose, we got 100 percent tumors of the dose of all of the animals, and there was dose response with regards to

both the latent period, as well as percentage of the tumors. And it was a pretty linear response until we got to 5 mg/kg and at 1 mg/kg. We got 40 percent of the animals. Again at 0.1 mg/kg, there were no tumors when the study was stopped. Minimally, this is only 10 animals per group, but it is administration of a very potent carcinogen, diethylnitrosamine, which at that top dose 40 mg/kg, we got 100 percent of the animals with a hepatocellular carcinoma. So, there is a clear dose response and a clear latent period over the lifetime of the animals.

DR. ATRAKCHI: Thank you. Dr. Rice, would like to commend on Question 3?

DR. RICE: Thank you. I am having considerable difficulty with the signal fading in and out here. I do not have specifics to add to this, but I want to express some concurrence with first the caution expressed about calculating the NOEL dose of carcinogenicity with a potent genotoxic agent like any of the nitrosamines under discussion. And I do not think it is really practical in a comparatively low-dose range to attempt to identify in-vivo exposure

that would rather absolutely define low versus high risk. I do not see how the continuum, that is response, can readily be found in these agents, except, of course, as the dosage becomes very, very much higher in any of those that would be experienced by anyone from taking any of the drugs that are under consideration. With that, I have nothing more to add.

DR. ATRAKCHI: Thank you. I would like to go back to one point we made here in the question, which is Ames negative. We use Ames negative as the first step or the first test to determine if a chemical is mutagenic or not. Some of those nitrosamines could test negative in the Ames test. This is difficult for us to decide that this would be acceptable, even though the Ames test was conducted properly under GLP in a valid test, but the result is negative. What would you recommend? Would you accept a single, well-conducted valid Ames test to conclude that a particular nitrosamine is negative, is not a mutagenic agent? Or would you follow this up with an in-vivo gene mutation test or any other followup mutation test to verify the negative result?

DR. ZEIGER: This is Dr. Zeiger. With regard to the negative Ames test for nitrosamines, the response on the Ames test for the nitrosamines is very species specific and very protocol specific. Initially, dimethylnitrosamine was reported as negative in the Ames test until people started increasing the relative liver homogenate concentration to VMN concentration, and then it became positive. Similarly, you have different potencies of responses when you test the same chemical using rat liver, mouse liver, or hamster liver, to the extent if you are using rat liver, which tends to be the least sensitive to the nitrosamines, you might get a very weak or negative response with rat liver but might get a fairly potent response if you are using mouse liver or hamster liver. We do not know which one of those livers is most comparable to what would be obtained with humans. So, as far as I am concerned, even a negative mutagenicity study with nitrosamines, if the structure is such that you think it might be metabolizable. And Dr. Eisenbrand showed a number of structures early on that you would not expect to be

activated to alkylating agents. You know, if the structure does not tell you that it should be or might be negative, I would consider a negative Ames test as not sufficient to say it is not going to be a carcinogen.

DR. ATRAKCHI: Thank you. Anyone else would like to comment?

DR. ADAMSON: I would think you would want to follow up with an in-vivo mutation assay before I would accept a negative Ames test as being possible.

DR. ZEIGER: I agree to some extent, but the in-vivo assays tend to be less sensitive, though it does not hurt to look at the in-vivo assays. They are getting better every day. We are now able to look fairly easily at gene mutation, as well as chromosomal mutations in red blood cells, mutations that were induced when the cell was still nucleated. So, a positive in-vivo assay would trump negative in-vitro assay. I agree with you on that. But because of the variations and responses with different protocols in the in-vitro assay, I would be reluctant

to conclude that something is negative based on a single assay with, for example, rat liver S9. Using the standard OECB protocol. The OECD protocol is a minimum protocol. It is not the definitive protocol.

DR. ATRAKCHI: Thank you. Anyone else who would like to comment on this?

DR. ADAMSON: Yes. The in-vivo mutagenesis assay picks up a number of compounds. I do not know about nitrosamines but some related to nitrosamines like dimethylhydrazine, which are negative in the Ames assay but are positive in in-vivo mutagenesis assays. So, it is a good followup, but of course, it is a lot more expensive and a lot more time consuming.

DR. ATRAKCHI: Thank you. So, I understand that even though not everybody responded to this question, but the general agreement is that a negative Ames by itself is inadequate to conclude that the compound is negative for mutagenicity.

There is one question from the attendees. I will read it. Would experts consider there is a limit maximum exposure for less than

lifetime and number of nitrosamines in a drug product? Multiple nitrosamines in drug products are not limited in M7. The less-than-lifetime allows 80-fold acceptable intake for 30-day treatment, so that theoretically, the total exposure for nitrosamines may increase to even milligram amounts. Would anyone like to comment on this?

I think that basically the question is what is a low-dose range for a nitrosamine exposure? Maybe that is what the question is.

UNIDENTIFIED PANELIST: Could I comment on that?

DR. ATRAKCHI: Absolutely, please.

UNIDENTIFIED PANELIST: Just as a pragmatic answer -- I mean I would personally orient myself on the unavoidable exposure from foods. The nutritional exposure is there, and I think it is in most cases, it is considered to be somewhat higher than the potential exposure from contaminated drugs. But of course, that has be check in any case. And if it comes to the mentioned dose level of milligrams, I would think this is in my opinion not feasible. It is

far exceeding the levels of nutritional exposure.

DR. ATRAKCHI: Thank you. Anyone else?

DR. KYRTOPOULOS: May I comment?

DR. ATRAKCHI: Yes.

DR. KYRTOPOULOS: Surely, it would depend very much on which nitrosamines we are speaking about. I mean the idea of affecting a milligram of NDMA even for a few days is just not something that one would consider. On the other hand, if it was something like a nitrosoproline, which I know is not the case for drugs -- I mean a noncarcinogenic nitrosamine, it would be a completely different thing. So, one has to look at specific cases. I do not think we can put -- it would be a case-by-case evaluation. I do not think we can put a general number on this.

DR. ATRAKCHI: Okay. Thank you very much.

So, we should probably now break for lunch. We will come back at one o'clock. Thank you very much.

DR. ATRAKCHI: Welcome back. It is 1:01. Before we continue to the last question of

today, Question 4, I would like to go back to Question 3 for a moment. It is fairly important for us as regulators to really understand a little bit better. With your responses and your thoughts and the reasons for your recommendations about Ames negative. The issue we face is that a nitrosamine will be tested, comes negative in Ames. They would repeat it. It would be repeated let us say with a blood product with a mouse or a hamster S9. The test becomes negative as well. So, let us address this scenario. Would that be convincing that this nitrosamine is negative in Ames? Is not a mutagenic nitrosamine and will end it there. And this means from a regulatory perspective that the next step is this impurity, nitrosamine impurity will be considered a regular impurity, meaning it will fall under ICH Q3A or B where it is controlled under much higher levels than an impurity that is mutagenic. So, it is a very important regulatory decision to make to allow such a one-test or two-test of a nitrosamine of a negative nitrosamine in an Ames test and then move it from the category of a cohort of concern to a regular impurity. We really

would like to understand from you your expertise and your knowledge of is this an acceptable or if one conducts an Ames with an S9 from the rat and it is negative, they follow it up with an Ames using a hamster S9, and it is negative, would they need to confirm because we may have missed something -- those tests have missed something -- and it has the nitroso group. It is on structural alert. Would this need to be confirmed in a followup test, whatever that test is? Whether it is an in-vivo mutation test or any other that from your experience would provide a more reliable result? Anyone who would like to comment?

DR. ADAMSON: I will start by saying I think that you need an in-vivo followup, not another in-vitro followup, regardless whether it is human liver, whether it is nonhuman primate liver, whether it is hamster. I think you need an Ames negative, and then you need an in-vivo assay negative, from my perspective.

DR. ATRAKCHI: Thank you. Anyone else?

DR. GUTTENPLAN: As I said before, there are certain compounds that are more sensitive --

DR. ATRAKCHI: Yes. Dr. Guttenplan?

DR. GUTTENPLAN: Yeah, as I mentioned before, there are certain chemicals that are more sensitive in the in-vivo metagenesis assay than in the Ames assay. And also in the in-vivo assays, you can give repeated doses over a longer period of time. So, the assay can be quite sensitive. As mentioned before, also, it is more time-consuming and more resource-consuming. And then assuming you exposed the animal for a sufficient time and sufficient dose and you get a positive result, what does that mean? That is another question. If you give enough of the compound for a long enough period of time, is that relevant to human exposure? So, that is just a question.

DR. ATRAKCHI: Now, when we are talking about in-vivo mutation assays from your perspectives -- there are a number of them. M7 has a number of them in their table over there in the Guidance. But clearly some of them are better than others and depending as you indicated on the compound itself, one would be preferable over the other. But,

which ones would you think for a nitrosamine would be more appropriate than other in-vivo mutation tests?

DR. GUTTENPLAN: I think for nitrosamines the Mutamouse assay, the Big Blue Assay. There is a Japanese assay. I think it is GDL Mouse. I am not sure of that, but that would also be another assay. Those are the ones that I am familiar with those, and those are pretty good for detecting particularly relatively small molecular weight lesions.

DR. ATRAKCHI: Very good. Thank you. Anyone else?

DR. HECHT: I think we have to consider the carcinogenicity database that we have for nitrosamines. There are very few nitrosamines that are noncarcinogenic, really only the nitrosamino acids with maybe a few other exceptions fall into that category. So, I absolutely agree with everything that has been said so far. Just a negative Ames is not enough. You need to do an in-vivo test. You need to have tested thoroughly before you can conclude that nitrosamine compound would be noncarcinogenic or

nonmutagenic. Thank you.

DR. ATRAKCHI: Yes. To your point, Dr. Hecht, and also to Dr. Guttenplan, your comment, what does it mean if it is in-vivo positive when the Ames was negative? You are correct, but from a regulatory perspective, the first test or group of tests, the battery of tests is mutagenicity. Ultimately, the concern is carcinogenicity for risk assessment. So, this is why it is essential, and it is important and critical for us to determine if the nitroso is mutagenic. It is the first step, and that is why we need to confirm if it is negative, why is it negative in a mutagenicity test. What we need to do is a followup, and that followup is an in-vivo mutation to further verify the organ metabolic mechanisms in place and an in-vivo system, physiological conditions to make us at least more comfortable in making the decision if that nitroso is negative in the in-vivo mutation. Ultimately, it is the carcinogenicity, but we cannot possibly continue with an impurity such as nitrosamine and go ahead and conduct a carcinogenicity, a two-year bioassay for every nitroso

that has been detected. So, we are trying to be reasonable and practical and resource-sensitive in what we ask for, but we also need to make sure that it is the public safety that is important.

DR. HECHT: Well, the first thing to do is to look at the structure and compare it to the huge amount of data that we have on structural aspects of nitrosamine carcinogenicity. You will not find many negatives.

DR. ATRAKCHI: That is correct. That is the concern.

DR. BUCHER: This is John Bucher. I think that if you are looking at doing an in-vivo assay after a negative Ames, which I agree is the best thing to do, I would encourage that there be a requirement that there be a couple of known nitrosamines running along with that assay, whatever that assay might be, so that one could (a) make sure that that particular assay is picking up nitrosamines, and that (b) you have some even imprecise idea of relative potency.

DR. ATRAKCHI: Absolutely. A positive

control of one of the nitrosamines, which likely would be NDMA or NDEA would be run in parallel in the same test. Anyone else from FDA who would like to comment on this or add to this question that I know we all are interested in having a discussion over?

DR. MCGOVERN: This is Tim McGovern from FDA. I'll just ask -- and I think it was Dr. Hecht who mentioned it -- that you are just looking at the nitrosamine database. There are very few nitrosamines that test negatively in a carcinogenicity study. So, I guess I would just ask the question, is there any concern even should one of these impurities test negative in an Ames assay or a modified Ames to some degree and then a followup in-vivo assay, would you still have any residual concern about its carcinogenicity potential?

DR. HECHT: I think if it was negative in the Ames and negative in an in-vivo system assay and you take a good look at the structure compared to what is known about nitrosamines of similar structure, I think you would be on solid ground to say that a particular compound would likely not show carcinogenic

activity.

DR. ATRAKCHI: Okay. If there are no further comments on this question, then I think we will move on to Question 4.

Okay. How would the risk assessment change when multiple nitrosamines are present in a drug product? What are the key variables to consider when conducting such risk assessment? One nitrosamine could be mutagenic carcinogen with another one that is mutagenic carcinogen and so on. This is not an unlikely scenario unfortunately. More than one nitrosamine has been detected recently in a single drug substance and/or drug product. Would the risk be additive or synergistic. Do we know how the in-vivo PK or pharmacokinetics would change when we have multiple nitrosamines in the same drug product or drug substance? What is the efficiency of the DNA repair to handle multiple nitrosamines at the same time and considering all of the other sources of nitrosamines together? I would like to start with Dr. DiNovi.

DR. DINOVI: Thank you. As with the questions we have done so far, this is a very

multilevel, multifaceted question. My particular area of expertise is on the exposure side. I'm the Dietary Accessor on the Center for Food Safety. And when we consider I will not say risk assessment since certainly substances added to food are not supposed to present a risk, but you understand what I mean. When we are looking a chemically closely related substances, our default assumption is that the effects, any toxic effects would be additive. And the way we deal with that is simply do the exposure in a way of simply adding the materials. More to the point of the nitrosamines here, though, there are classes that come back to my mind, where the structures present different toxicities, and what our toxicologists have done as have others around the world is taken toxic equivalent factors into effect. So, the way you deal with it in the assessment, of course, is you look at the exposures and you weight the exposures based on the relative toxicities. It is fairly straightforward and fairly simplistic, but we are also not looking at situations with carcinogens typically. These are in fact, but you are not looking

at the kind of questions that we have here where it is nonmutagenic versus mutagenic. That part of the answer to this question I am going to have to leave to our toxicology colleagues, and we can come back if there are other questions we want to talk about with the exposure. So, let me just pass it on at this point. Thank you.

DR. ATRAKCHI: Dr. Cronin.

DR. CRONIN: Thank you, yes. And I am going to put a modeling slant to this. So, I think the previous speaker set me up quite nicely in many ways because I was going to start off by saying, well, really repeating the question here. The key consideration is whether the concentrations, the potencies are additive and whether we can make an assumption as we have just heard or what would we need to assume synergy. I will start off by also passing the buck on the synergy question. I am not aware of synergisms specifically in carcinogenicity. As I say, I will rely on the experimental toxicologists who determine whether I am right or wrong or whether we need evidence on that. I guess where that may come in

place is if we see things like increased metabolic activation or knocking out of defense mechanisms. And again, if we are going to assume that from my simplistic modeling point of view, if we are assuming that some of the defense mechanisms are relatively generic and unspecific, then we can probably assume additivity. I agree with the first respondent. We will consider similar structures. We will work together and beat the additive. But we are assuming in that the similarity in mechanism, similarity in potency, and similarity in reactivity. This is quite possible, but also let us bear in mind the subtleties of some of the reaction mechanisms, and we know we have these what we know as activity cliffs when we have the correct substitution patterns. So, here we also have a second opportunity to think about categories and chemicals and when can we lump together, when can we group together molecules and understand the problems. And also where do we have the data. And I am going to discuss data more tomorrow in the answer to Question 6. But we need to consider it is not just a question whether we can

group together, but do we have data to support any argument. And also think about the differences. We are actually getting quite good in some areas. I will talk again in more detail about read-across tomorrow, but we are getting quite good at understanding differences between molecules and what they may potentially have. Here we can understand them in terms of reactivity and possibly bioavailability.

So, we also need to think about the mechanisms of action and whether we have information of mechanisms of action. And we have also been thinking, wondering what scenario would be if we had for instance a very high potency carcinogen and something that may be acting by the same mechanism but with a much lower potency. There is really a need in that regard to even include additivity if the high-potency carcinogen is several orders of magnitude above that of the low-potency carcinogen. More specifically around read-across and QSAR. Again, I will define these terms and talk about them a little bit more tomorrow. Let us start with QSARs. I am not aware of an QSARs for mixtures with regard to these

particular endpoints. Mixtures related to QSARs tend to be on the whole the vast majority for acute toxicity or acute lethality. Where we do see occasionally additivity on a very occasionally synergism. In terms of read-across, again read-across relies on adequate data. So, here would be adequate data for two or more nitrosamines that we could extrapolate across to a set of similar compounds. We will talk about this tomorrow. It is possible. It has been done. There is some work on read-across for mixtures, particularly within the UVCB area, but is really assuming that we can assure ourselves of the similarity of our structures. So, we are again getting back to this argument of structural activity, and we know we have some knowledge in that area.

With regard to the question, one area where we can use SAR and QSAR -- we have just been having this discussion of course -- is to predict whether a compound may be mutagenic or may be a nonmutagenic carcinogen. So, we may be able to take if we have two or a small number of nitrosamines in the sample, we may be able to use particular modeling

approaches or read-across to enable some kind of assessment to be made to at least see whether or not they fit into those categories that are on the screen there.

The other thing I would like to highlight is the possibility of using some of the techniques that are currently applied in terms of commutative risk assessment, particularly commutative assessment groups. And the European Food Safety Authority, EFSA, has done a lot of work in this area. And their work is on residues from pesticides. So, not nitrosamines but in some ways analogous to what we are talking about here. So, I think there could be some learnings from that, and they have a full-stage methodology based around identification, characterization, collecting the data, and grouping. And if you look at their approach, in part of their approach, they are saying at the lowest level where you have the least certainty, you can assume we are looking at some kind of structural similarity or grouping basis, but we may not know for instance, mechanisms of action or similar through up to the

highest level where we can assure similarity in mechanisms of action. So, as with many of the arguments in this workshop that I will put forward, it comes out to structured activity relationships and also being clever about what we are doing and learning and also building up bodies of evidence. And also thinking about how we can extrapolate what we are talking about in earlier questions about structural activity and showing that we are using the same information and the same groupings, not only for single chemicals for applying for single chemicals, but can we use that to apply up to groups of chemicals that may be present in the same sample. Thank you.

DR. ATRAKCHI: Thank you. Dr. Bucher.

DR. BUCHER: Yes. So, my answering this question really relies on the experience that we have generated over the course of many years looking at combinations of chemicals in toxicology studies, and it has really been our experience that irrespective of the mechanisms in general for these chemicals, additive models more than adequately predict outcomes in the vast majority of cases. In

particular when you are dealing with chemicals of similar mechanism as you would think in nitrosamines and also certainly at the levels of nitrosamines that would be appearing individually in any of these drugs at an acceptable intake level, so I do not think it matters really to my mind. When we do chemical mixture studies, it does not matter whether they are mutagenic or not mutagenic, if there is a carcinogenic potency associated with a particular chemical, those would be the numbers to use for the acceptable intake level, and in my mind, it is -- so in my mind, in our experience, until you get to significant exposure levels of chemicals, you very rarely will run into anything that looks like either synergism or antagonism. So, I think additive models are probably more than adequate for the cases of nitrosamines that you are going to be dealing with.

DR. ATRAKCHI: Thank you. Dr. Guttenplan.

DR. GUTTENPLAN: Yeah, I basically agree with the previous comments. The important factor here is that we are dealing with subthreshold

levels with respect to DNA repair. As Dr. Kyrtopoulos mentioned, even for the saturable enzyme, which is not really an enzyme but a protein 06-methylguanine transferase, we are apparently well below the threshold in almost any organ of the body, and the levels in drugs is so low that we are probably not going to approach the threshold. I will say though that different nitrosamines are going to be repaired by different enzyme systems. Most of them can be repaired by the 06-methylguanine transferase system, but as you get to larger adducts, particularly above the ethyl group, then there are other base excision repair and nucleotide excision repair. And they are all going to play a role, but in each case, if you are subthreshold, then there is no reason to think that you are not going to have an additive response for each agent. So, I basically agree with what has been said so far.

DR. ATRAKCHI: Dr. Adamson.

DR. ADAMSON: Generally, within the same class of compounds, the default position is generally addition unless there is some indication

otherwise. But I would say -- you brought up additive or synergistic, there is also the possibility of less than activated, particularly when compounds need to be activated when you have binding and when you have DNA repair. But I would say at the low levels that the nitrosamines are present in a drug product, probably those do not factor in. You probably would not have competition for activation. You probably would not have competition for binding. You probably would not have competition for DNA repair. So, with regards to the small amounts that are present in drug products, I would say that you would probably, unless indicated otherwise, you would have to do an additive.

DR. ATRAKCHI: Thank you. Dr. Hecht.

DR. HECHT: Yes. I agree. Considering the low levels that we are going to be observing, additivity is definitely the default assumption of the molar amounts that are present. So, I agree with everything that has been said about additivity.

DR. ATRAKCHI: Dr. Rice.

DR. RICE: I too agree that additivity is the most likely way to consider the issue of

multiple carcinogens. I would think though that there is always the likelihood that if there is more than one nitrosamine in a product, it could well be one about which next to nothing or absolutely nothing is known. And I should not think that the regulatory focus would be to be undertake a full-scale search of chemical structure to identify precisely what this unknown nitroso compound is. I should think given the fact that it is fairly clear that dimethylnitrosamine or perhaps the most potent of known carcinogens, that an overall analysis of total nitroso compounds present in an adduct could -- from the standpoint solely of other health protection, you could treat them as essentially an equivalent total dimethylnitrosamine. Most of the time that will be an overestimate of the potential hazard, but that is an error on the correct side of caution. So, in sum, I would just treat them as additive, and in the case where there is a new unknown or normally known agent in addition to one of the better understood nitrosamines or something added to them as though they were equivalent in the known publications. Thank you.

DR. ATRAKCHI: Thank you. Dr. Kyrtopoulos.

DR. KYRTOPOULOS: Yes, I think Dr. Rice made an excellent summary of the situation. And I agree 100 percent with what is being said. Basically, additivity and if necessary, taking the potency of the most powerful of the nitrosamines, NDMA. I do not have anything else to add.

DR. ATRAKCHI: Thank you. Dr. Eisenbrand.

DR. EISENBRAND: (No response.)

DR. ATRAKCHI: We can move to Dr. Zeiger.

DR. EISENBRAND: So, I am back. Sorry. I had a lot of problem with it. Well, I agree totally with what has been said concerning additivity. I think an additive modification be applied here, and it may be in a case where we have nitrosamines with vastly efferent biological activity, the most potent ones and nitrosamine of minor potency, then I would think that the potent one, of course, would primarily add and be the one that should be looked at and

evaluated. Just to mention, there has been I think it was in 1990 a publication from the German Cancer Research Center, the first author I think if I recall correctly -- I can send you this -- is Pergot [ph] where they tested combined application in rats of I think it was diethylnitrosamine or dimethylnitrosamine, nitrosomorpholine, and nitrosopyrrolidine, and what they found out in that lifelong exposure study was clear additivity of the effects. So, that is published already since many year.

DR. ATRAKCHI: Thank you. Dr. Zeiger.

DR. ZEIGER: Okay. I also agree that additivity is the most appropriate way to go for the reasons expressed by all of the previous speakers. Obviously for the data just presented. Just one point on the question. I would not separate out mutagenic from weakly mutagenic carcinogens. I agree with the comments on additivity. Thank you.

DR. ATRAKCHI: Thank you, Dr. Zeiger. I would like to make a comment here. Well, to sum up this question is basically everybody seems to agree

that if we have one nitrosamine as a contaminant, as an impurity in the drug, will we have five? To be the most conservative approach is to use the amount of the most potent nitrosamine and to apply for all the five, even though there is one or more of the others that either there is no carcinogenicity data or the data is very poor. And it seems like almost -- well, actually all of you agree with this assessment that the risk assessment would be an additive in using the most conservative and most potent carcinogen, and nitrosamine is carcinogenic.

Going back a little bit for the whole day that we have been discussing now, reaching to Question 4, it would seem to me that all of you have indicated the large amounts of endogenous formation, and that is due either internally formed or exogenously from foods that we are exposed to for nitrosamines. And the miniscule amount that we could be exposed to from drugs. I assume you are not saying or indicating that we do not need to worry about the small amounts of nitrosamines in our drugs. Is this accurate? And certainly, that is not the case. It

does not seem that is the case based on what we just discussed in Question 4 where we said if we have one or more nitrosamine, we need to use the most conservative limit of the most potent carcinogen, and use that one as the total for the five or six nitrosamines in a drug. However, it is not clear to me that from what we have discussed all day that it is the endogenous formation, the exposure from exogenous intake compared to the minimal or the small amount that has been contaminated in drugs should be an issue. Is this what you have indicated or not?

DR. EISENBRAND: Maybe I comment first, if you allow.

DR. ATRAKCHI: Absolutely.

DR. EISENBRAND: Well, you know, as I pointed out in my lecture, the data on exogenous exposure first in my opinion are relatively outdated. So, it is our suspicion, our interpretation of the data with our approach that the amount that is being taken in for contaminated drugs by comparison may be very, very low. But we do not know really for sure. That is my point. That is why I think we need to

update first the database on exogenous, but even more so the database on endogenous formation of nitroso compounds. We need really scientifically based updates on these data because they will become very important when it comes to risk assessment. And I mean I just recall the last one of the publications about ranitidine I show where you have this publication of 2,016 reporting about substantial in-vivo formation of nitrosodimethylamine from ranitidine. We need to look at that. This is a very relevant question in my opinion still. By and large, I agree with you that probably we finally can say that normally the contamination with preformed nitroso contaminants of drugs is relatively negligible in comparison to the exogenous exposure for food and even more so to the endogenous exposure. But we need to have safe data on that. That is my opinion.

DR. ATRAKCHI: I understand that there are recent studies that came out of the Center for Foods where they indicate the amounts in foods are fairly low. The amount of nitrosamines in foods over the past 20 years is extremely low. So, we need to

take that into consideration, and I think Dr. DiNovi can comment on this.

DR. DINOVI: Yeah. It is certainly less than 1 mcg a day, and that is true for all of the studies we looked at. One of my colleagues, Dr. Jolie [ph] just recently went through and did a review of nitrosamines in food, and that was the conclusion, less than 1. Even at a high percentile of the distributions. The thing about what we are talking about here is precision in a risk assessment is much more of an academic than a regulatory concern. At CDER, you need to make timely resource-efficient decisions, and we really do not have the luxury to go beyond some of these false assumptions.

DR. ATRAKCHI: And also in terms for drugs, the drugs they have a GMP. They need to be clean and not containing impurities. So, it becomes an issue of quality. At the beginning, it is a quality issue. Then, it comes the safety issue. So, that is important for us. We cannot allow any impurity, whether regular impurity or mutagenic impurity to be above a certain level. That is why we

have guidelines. That is why we have regularity limits on such things, excipients and impurities and so on. But beyond that, the nitrosamines as we all know, they have been put in its own class as a cohort of concern in N7. They need to be much more restricted below a 0.5 mcg per day because of their potency as mutagenic carcinogens. So, it just seems to me that whether it is this study, as you indicated, Dr. Eisenbrand, the database needs to be updated. There are some new studies that did show there are very little amounts of nitrosamines in foods, and the intake is fairly low. So, I am just trying to make it clear that none of you is saying that we do not need to regulate or we do not need to worry too much about the small amounts of nitrosamines in drugs. I am assuming that is not what you are saying here.

DR. RICE: May I make a comment on that?

DR. ATRAKCHI: Absolutely.

DR. RICE: I found in reading the materials you supplied in preparation for this workshop, both in the Guidance document and the

European Medicine Agency, very brief mention of laboratory findings that the amount of dimethylnitrosamine in ranitidine samples tended to increase over time, and thus could not very well have come from contaminants arising in the manufacturing process. If in fact you look at the structure of ranitidine, which is I suppose fairly remarkable in that within the molecule there is both a dimethylamino group and a potentially nitrosating nitroalkene structure that dimethylnitrosamine may be forming in the finished drug product as a decompensation product that tends to increase over time. And I would very much like to see that suggestion confirmed or refuted by further studies. If a nitrosamine contaminant analyzed during decomposition of the active pharmacologic ingredient, then that renders almost moot efforts to calculate just how much is derived from whatever might have been there at the beginning of packaging or whenever a single sampling was done. Can you comment from the FDA standpoint on this issue?

DR. ATRAKCHI: Yes. I will let Dr. Keire perhaps can comment on this.

DR. KEIRE: Yeah. Sure. I think the drug has to be stable over its shelf life and keep any nitrosamines that may be forming over time to a level that is acceptable. And when we looked at ranitidine samples over time -- I am talking about months -- that we observed that the amount of NDMA did increase over time, and they went above the viable intake limit that had been set for the drug. Of course, this was part of the information that was used to make a decision about requesting market removal of the drug. So, that is one aspect of it. But I would also mention that there is a recent publication from the group that showed that this was very formulation-specific. So, if you have particular polymorphic forms, amorphous versus crystalline forms of the drug, you would get more degradant in one form over another. So, it is possible, and we also observed this in other, from product to product, the amount that you would see that would form over time would be very different. Some products remain below this 96 nanogram limit for long periods of time. So, there is a potential that there are some formulations that could stabilize this drug

such that the amount of NDMA would not form excessively. And I guess the other thing I would like to comment on is the very valid point brought up by Dr. Eisenbrand about, you know, these studies that are about endogenous formation of NDMA in the GI tract, and I guess where the supposition is that this is happening, and the FDA is also concerned about that. And whether there is a clinical trial that has been performed to look into exactly that point. That has not been completed yet, but certainly we are very interested in checking the results. Like you said, what we really be careful about what we base our decisions on. We need to have very good data and know what has been discussed. The scientific literature in this area is fraught with examples of measurements gone awry for whatever reason. So, we have to take a lot of care to measure things down here in the parts per million and parts per billion range, and certainly the sample preparation is key. You do not want to introduce any artifacts in the measurement process itself. So, I will stop there. Thank you.

DR. ATRAKCHI: Thank you, Dr. Keire.

Anyone else who would like to comment?

DR. ADAMSON: I generally what you say was correct, but I also agree that we do need some up-to-date data with increased measurements to see what endogenous formation of the nitrosamines is. And I would also like to bring up I remember reading several years ago a study I think it was by Shubik where he gave -- I believe the compound was pipercycline, but I am not positive of that. But I am probably 90 percent sure. It was formulated with ascorbic acid N. And therefore nitrosamine was not formed. But if you checked it out without ascorbic acid being present, you would be forming in the same laneu [ph] as in the stomach that could be forming a nitrosamine. So, I think there is something positive to consider formulations of some of these drugs with an antioxidant, like the vitamin E or ascorbic acid or something else. And I think also with regards to the comment that came up with regards to the final formulation of a drug, it might be interesting to check out the final formulation, which does or does not contain an antioxidant to make sure that the drug

does not break down to nitrosamine. Thank you.

DR. EISENBRAND: Could I comment as well?

DR. ATRAKCHI: Yes, please.

DR. EISENBRAND: It is very right what has been said. This compound, ranitidine, reminds me very much of aminopyrine, the compound I showed in my talk where we literally found that this always just presents a snapshot of dimethylnitrosamine contamination because you could not predict it because any time you measure it again, it turned out to be higher. And Dr. Keire has already alluded to the problem of these highly reactive materials of avoiding artifact formation during analysis. This is not trivial. This is a real problem because one has really to try everything to show that there is no artificial formation. But by and large, these compounds with these structures like aminopyrine or I think also ranitidine are really very easily reactant. And that is why I think this potential of in-vivo formation needs to be really considered thoroughly. And in a sense putting antioxidants or ascorbic acid

into the formulation may be a very good possibility. But with aminopyrine, we measure this also using ascorbic acid to inhibit dimethylnitrosamine formation. And it turned out that the pharmacokinetics are quite different. For instance, aminopyrine was recirculated for the gut salivary glands, reflecting just blood levels, plasma levels. And ascorbic acid was after the first passage, it was just done. So, the protective action of vitamin C was just for the first passage through the gastrointestinal tract, not for the delayed one when the drug was recirculated. Maybe it is different with cimetidine but maybe not. So, that was my comment.

DR. ATRAKCHI: Thank you. Any other comments?

DR. KEIRE: Yeah. I mean I guess that each of these drugs is different, and so there is going to be some case-by-case analysis of the reactivity of these things and the conditions needed to get conversion of any particular drug. So, there is nitrosation chemistry. There is a lot of literature about it that is quite complicated, and the

conditions have to be right to get certain reactions to go. And so, I think that is another consideration. But in basic chemistry, there is not the potential for particularly biologic enzymatic processes that might lead to formation of nitrosamine.

DR. ATRAKCHI: There is a question from the attendees. I will read it. Nitrosamine exposure level from foods including water relative to IV drugs, where IV drugs are not subject to digestion and absorption. Would anyone like to comment on this?

DR. EISENBRAND: Well, my comment would be that needs to be studied case-by-case. If you give a drug IV and it is just distributed systemically, it might very well end by being recirculated like many drugs where you can even measure blood levels in saliva. So, it really -- at first it will go through the gastrointestinal tract. The second point is that nitrosation may very well occur elsewhere in the body. As soon as inflammation or infections, it is always then connected to the generation of nitrogen monoxide and NOx. And then you have the nitrosating there. So, irrespective of the way of ingestion.

DR. ATRAKCHI: Thank you. Any other comments on Question 4 from the panelists?

I would like to go back to the biomarkers. We have talked a lot about DNA adducts and other biomarkers that could be used to determine risk assessment. Can we elaborate on this a little bit more? Can we have more discussion on what do you think about all of biomarkers that you have discussed, we have discussed today, that could be more appropriate for nitrosamines?

DR. HECHT: I think DNA adducts would be good to look at. I think that we have the technology now to reliably quantify DNA adducts by high-resolution mass spectrometry, and we also have the knowledge based on years of study about artifact formation. So, I think with regard to the question of endogenous formation, which is critical here because there are really high levels in endogenous formation, maybe we do not have to be that concerned about the low levels that are present in drugs. And I think we could envision experiments similar to what was done with nitrosoproline 25 years ago by looking at its

levels in urine when you gave people proline and nitrite. You can now envision studies where you can look at DNA adduct formation from compounds like dimethylnitrosamine by giving the precursors dimethylamine perhaps labeled and then determining the level of DNA out of formation using the labels to trace it. So, there are ways that you could really address this question. Dr. Eisenbrand mentioned still a critical question with respect to the overall exposure to nitroso compounds.

DR. ATRAKCHI: Is it fair to say that we really understand the mechanism or action of nitrosamines at this time? Do we really know it is only 06? Is it combination of 06 and N7 methylation when we say we can use the biomarkers let us say for the 06 methylation, would we be confident that this would address?

DR. HECHT: Well, I think we are confident that DNA damage is critical. I think we are pretty confident about that. We are reasonable confident that 06 methylguanine is important in the case of dimethylnitrosamine. And there is plenty of

data on that. But I you were going to look at DNA adducts, you would not have to restrict your analysis to 06 methylguanine.

DR. ATRAKCHI: Very good thank you.

DR. KYRTOPOULOS: May I add something?

DR. ATRAKCHI: Of course.

DR. KYRTOPOULOS: Certainly, 06-methylguanine is important, and there is a lot of experimental evidence from certain animals, directly modified animals, which clearly demonstrated carcinogenesis by methylating agents. However, just because it is the best studied model, we should not say that it is a general model. Not all nitrosamines methylate. In fact, I think that for many of them, I personally do not know which is the most important kind of DNA damage that they cause. So, DNA adducts in general -- first of all, I agree absolutely what was said before by Dr. Hecht regarding the need to apply modern, powerfully sensitive methodologies, clinically specific methodologies to the analysis. And certainly there is a potential today to go down to very high sensitivities. So, measuring DNA adducts is

a good way to have biomarkers of exposure, but if we want to go a step further in a mechanistic sense involving biomarkers of risk if you like, like 06-methylguanine is both a biomarker of exposure but also a biomarker of risk. We have to know much more about how the mechanism of carcinogenesis by many of the nitrosamines, which I do not think we do. Even for the simple ones. Diethylnitrosamine. Secondly, 06-methylguanine is important. But 04-artathiamine [ph] probably plays a role in some cases. In fact, if know the correct name -- it is a long time since I read this literature. I think 04-lithothiamine [ph] was accumulated. So, it could be that there are different adducts playing an important role in carcinogenesis by different nitrosamines. From what I am aware, we do not really know which are the key adducts. So, we cannot really say today that we know enough to develop risk biomarkers. DNA adducts is not exposure, quantitative exposure, or even just to verify that there is exposure. It is certainly important, and it is achievable today. That's all.

DR. ATRAKCHI: Thank you. So, you're

saying that essentially, we should not limit ourselves to only the 06-methylation, although for NDMA and for the nitrosamines that are well studied, we know that. We know that very well. A lot of studies have been done and shown that, but we should not be limited to only the 06 as our biomarker for exposure.

DR. KYRTOPOULOS: Yes, I think so. I mean the fact that NDMA is a problem in the context that we are discussing, the drugs. And it is a very powerful carcinogen. So, 06-methylguanine is certainly relevant to our efforts to evaluate the problem that this NDMA contamination poses, but I dare say, I would not generalize the importance of 06-methylguanine.

DR. ATRAKCHI: Thank you. I would like to ask my colleagues from FDA if they have any comments or if they have received any questions from the FNDs because I do not see any at this time.

DR. KEIRE: I guess I just have one comment. I think that there is a lot of DNA around, right? If you have a meal of fish -- I was just reading this -- there can be up to like 40 mg of DNA

in a meal. And certainly if there are conditions that are conducive to a nitrosamine formation, there is a substrate there pretty much all the time for it to happen. So, I guess it goes back to that conversation about what potential endogenous sources. And if the conditions are appropriate for formation of a lot NDMA from dimethylamine, which is a much simpler substrate than many of these drugs. Just a comment.

DR. ATRAKCHI: Thank you.

DR. GUTTENPLAN: I have a comment. In the publication with ranitidine, they monitored dimethylnitrosamine in the urine, but they also monitored total nitrosamines. So, is urinary analysis a possibility because this could be done in humans, in human volunteers who are taking the drugs anyhow. Just a question.

DR. KEIRE: Yeah, that is exactly right. The FDA actually conducted a small trial, and the results are now pending. So, hopefully, we will have new data on it to share on that soon on exactly that question.

DR. KYRTOPOULOS: May I add a question

to this? One thing that puzzles me about the paper is the following. I've seen few data on the levels of dimethylnitrosamine that have been found in drugs. The first analysis on ranitidine, the amount of NDMA that were found were enormous. I think it was 2 or 3 mg per tablet. Is that a real finding, or was it maybe an artifact because subsequent numbers were much lower. Because if that was the real level that present in the tablets that were taken by the volunteers who had these high levels in their urine, it might even be that they were actually taking it --

DR. KEIRE: Right. So those measurements were done by using a technique called headspace UCMF that using high temperature to vulcanize the sample for analysis. And ranitidine is temperature sensitive. I will form NDMA. So, those original reports of 3 mg quantities from ranitidine are not effective measurements, and we were speaking to that point earlier that you really have to be carefully how make your measurements on. Sometimes these compounds -- you know, and actually that was an FDA method that they used that was developed for

valsartan. So, it was developed for a different drugs, which was not heat labile, and they applied it to ranitidine and unfortunately, that led to this artifactual finding. So, what the FDA reports on its website are much lower numbers, still unacceptable numbers, but in terms of limits that are set right now, but much, much, much lower than was in those original reports.

DR. GUTTENPLAN: I believe they published the correction. The group that published the ranitidine they published a correction taking into account the head-space formation, and it was very minor. It was not a major artifact.

DR. KEIRE: So, I'm talking about -- there are two different things. So, there was a report by this private lab that reported these 3 mg amounts being formed. But you are correct in the Zang and Mitch [ph], they did work to see what percentage of the total from the measurements they did. It was only 5 percent formed of these large amounts that they were seeing was formed from the headspace. You have seen this method in their

application of it. But in the other application that was reported were these really very high amounts reported. That was just because of the headspace method used. So, two different things were being discussed.

DR. GUTTENPLAN: Okay. That's clear. Yeah.

DR. ATRAKCHI: Thank you. I cannot follow the questions on the side. They are moving on me very fast. But I would like to ask one question going back to Question 2 on the classification, and then maybe I will ask my colleagues to read some of those questions on the side. They disappear quickly from the chatroom.

In terms of the classification, EMA had come up with one value, one class, a specific number, which is 18 nanograms per day. Would that be something that you treat all the nitrosamines as impurities with one number, and that is lower than the most potent nitrosamine, NDEA, which we have listed as an acceptable intake of 26.5. Is that something that you would consider? Anyone on the panel?

DR. EISENBRAND: May I?

DR. ATRAKCHI: Yes, please.

DR. EISENBRAND: Actually, I wouldn't really think there is a big difference between 18 and 26. In terms of biological efficacy, it is the same ballpark more or less. So, these default values of extrapolation of potential acceptable mutates in my opinion are helpful and could be used as a primary measure to protect actually the consumers, but in fact we need scientific confirmation of this. And this, I think I come always back to the proposal that one should really look into what happens outside in the real world, which, in my opinion, is just what we need necessarily to take up with foods. And admittedly as we discussed before, this is low. It is below 1 mcg a day, internationally even. But by comparison to the exposure to the potential exposure for contaminated drugs, it is still higher, much higher in most cases. I do not know of all of the cases, because this is a problem that rapidly develops. Many things I just do not know. I am not aware of. But as a pragmatic proposal, I think this is definitely in my opinion a

rationale to go on and pursue what is happening outside the drug situation to compare with. It is more or less the levels of nutritional uptake of at least -- it is below 0.3 mcg per day. There is at least a good orientation.

DR. ATRAKCHI: Thank you. Any other comment on this?

DR. CRONIN: Yes, maybe if can just add a thought. I certainly agree it is a starting place. And we have heard the arguments for that. Clearly, you may look for evidence and go to a higher level if such evidence exists. I am certainly aware of some work from Kevin Cross who recently presented some information that suggested that you can use SAR and read-across. We will talk more about this tomorrow. In some circumstances, we can do read-across and demonstrate the very high probability that you could go to a higher level. So, that is how I would view it, that if you had no other data or information, then you would start there. Then start to build your lines of evidence. And certainly starting with SARs and looking for similar structures in similarity in terms

of mechanisms and seeing whether you could read-across and whether there was room to increase that in terms of safety. Thank you.

DR. ADAMSON: I would like to bring up one factor that has just been touched on, and that is are there sensitive populations. For example, ranitidine was in a syrup that was used primarily for children and infants. Are there other examples where there is potential nitrosamines in such medicines that would be given to newborns because I think they should be more classified as perhaps more prone to develop adverse effects than adults.

DR. ATRAKCHI: Are you suggesting that one number -- let us say it is the 18 nanogram per day would need -- maybe we would need to apply safety factors for the more sensitive population because that is certainly not in ICH M7 because ICH M7 considers that the values in the assessment is fairly conservative, that there is no need to add safety factors for pregnant woman or children or any other sensitive population.

DR. ADAMSON: I am not phrasing the

issue. I think it needs to be looked out.

DR. ATRAKCHI: Okay. Thank you.

I will read this comment or question. I think that the discussion of the Ames test appeared somewhat contradictory. Could we get some clarify from Dr. Zeiger's statement that the common LACD protocol was insufficient and that it needed adapting, e.g., additional S9 systems, or was the overall conclusion that even a modified assay would be insufficient.

DR. ZEIGER: I did not mean to imply that there were problems with the OECD protocol. But the OECD protocol, you know, allows for a variety of options. For example, I have seen many labs that do the Ames test with 5 percent S9 as an example. This is acceptable within the OECD protocol. Yet something like dimethyl- and diethylnitrosamine need much higher levels of S9 in order to respond to the assay. This is what I mean. FDA OECD protocol limits the strains that are used for testing. There are other strains that will be positive, whereas these strains might be negative. The OECD assay protocol emphasizes the rat

S9. I think there is more than enough data to show that other S9s, other rodent S9s, are more suitable for nitrosamines than rat S9. So, if I got a negative for a very weak response with rat S9, I would immediately go to the mouse S9, which might be much better or hamster S9. Or a higher concentration of S9. These would still be allowed within the OECD protocol, but most laboratories will not do this in general. I would not do it if I was looking at polycyclic aromatic hydrocarbon, but I would do it if I was looking at a nitrosamine that came up equivocal or negative.

DR. ATRAKCHI: Thank you. I agree. There is a great deal of literature out there to show that the rat S9 may not be sensitive to the nitrosamines, and some modification in the test system in the Ames needs to be conducted in order to provide the more appropriate response.

The other comment or question. I do not think the panel answered this part of the Ames discussion. If an Ames assay is not considered conclusive, then why would not the panel recommend

going directly to an in-vivo assay directly? Anyone would like to answer this

DR. GUTTENPLAN: Yeah, because it would be too resource consuming. It is a lot more work and probably orders of magnitude more expensive. So, if you can already find a positive in the Ames assay, you do not have to go ahead to he in-vivo assays.

DR. ATRAKCHI: You are correct.

DR. ZEIGER: Based on the available data, a negative in-vivo assay does not counteract the positive Ames assay. They have many chemicals other than nitrosamines that are strongly positive in Ames tests but negative in in-vivo, but gets still negative in carcinogenicity. Well, it is still positive, I am sorry, in carcinogenicity. So, if you have a positive Ames test, no other test really negates the implications for carcinogenicity of that positive Ames test, no other gene tox test that we know of.

DR. ATRAKCHI: Very good. Thank you.

What are the possible reasons a negative Ames test when it is positive in the in-vivo?

DR. ZEIGER: Well, the Ames test does

not detect every type of DNA interaction, every type of DNA damage. The Ames test will not detect DNA deletions, which might still allow survival of the cells. But the in-vivo tests look at different endpoints, the same endpoint but different target sites and have different sensitivities. So, something like the new Pig-a test -- that is a gene mutation measurement in blood cells -- will detect deletions, whereas as an Ames test will not detect deletions. The Ames test does not detect all possible DNA damages.

DR. ATRAKCHI: Thank you.

DR. GUTTENPLAN: There are compounds that are just not metabolizing well enough in the Ames test but are metabolized more efficiently in-vivo, so you will get a positive result in-vivo. I think something like diethanolamine will probably not be positive in the Ames test, but I will bet under the right conditions, it will be carcinogenic and mutagenic in-vivo because there is more metabolic capacity. Many, many years ago people were doing host-mediated assays, and there were compounds that

were negative in Ames test but were positive in the host-mediated assay. The reason was in the host-mediated assay where you injected the bacteria into the tail vein of the animal and then recovered the bacteria, you had the whole liver metabolizing the carcinogen. So, that is another reason why the Ames test does not always detect a potential mutagen is there just is not sufficient metabolic capacity.

DR. ZEIGER: Yeah. I would like to support that point. In the Ames test, we are just looking at the metabolic capability of a liver homogenate supplemented with NADPH, whereas as was just said, in the animal you have the intact liver and other organs doing the metabolism. Though, I would walk back the statement on the host-mediated assay. That is the assay that got me involved in this deal. Then, my Ph.D. dissertation was on the host-mediated assay in nitrosamines. In theory, it is a very sensitive test. In practice, it is a very insensitive test because it is measuring mutation in bacteria in the peritoneal cavity of the animal, which means for something DMN or diethylnitrosamine, the active

metabolite actually had to get into the peritoneal cavity to deal with the bacteria, so you are working with a very low level of active product, and very few chemicals were mutagenic in the host-mediated assay, except for some of the cyclic nitrosamines.

DR. EISENBRAND: May I just comment for second on the data because it was just mentioned by Dr. Guttenplan. In diethylnitrosamine is a medium potency carcinogen, by far not as potent as NDEA for instance. And we have investigated the mutagenicity response to the Ames test. First of all, it was not really active, but it became active when it was activated with alcohol dehydrogenase. And this was published years ago. But later on, they also found that is activated by alpha-C hydroxylation. So, we have both activation processes. One is the beta oxidation of the OH group or groups, and the other one is the still ongoing alpha-C hydroxylation that finally turns the compound into a DNA alkylating agent with a positive mutagenicity test.

DR. ATRAKCHI: Any other comment on this?

Okay. I would like to go back to the use of TD50 versus the BNDL. Could we have more discussion on the pros and cons of each? It seems like the tendency was more preferable for the BNDL.

DR. BUCHER: This is John Bucher. I can address that. So, the main difference is the BNDL uses dose response information, whereas the TD50 uses a point estimate to extrapolate to a particular risk level. Anytime you are dealing more doses, you are dealing with better precision as to a lower dose estimate of risk. But the problem is that the calculations require multiple dose groups, and for the nitrosamines, there are many nitrosamines in the TD50 or the carcinogenic potency database that perhaps do not have this kind of information, and certainly the calculation for BNDL have not been performed on the original data. So, I do not think anybody is arguing that the BND is less preferable than the TD50, but it is just sort of a practical matter of what is available.

DR. ATRAKCHI: So, you would use either one?

DR. BUCHER: Preferably, I use the BND if it is available.

DR. ATRAKCHI: Okay. Another other comment?

DR. ADAMSON: I agree with John on this. If it is available, the TD50 is sort of a default. But I you do not have a dose response.

DR. EISENBRAND: Yes. I would also largely agree. I think the BNDL is preferable wherever applicable. Do not forget one could read-across to make the argument that a compound that has not the right data density still could be evaluated in that sense if it is close to the reference compound in structure that may be in TD50.

DR. ATRAKCHI: Thank you.

A question from attendees. If some nitroso impurity is unavoidable and it comes negative for mutagenicity in Ames, will in-vivo assay need to be conducted to follow up with an in-vivo Comet assay or a Pig-A mutation assay or transgenic mutation assay?

DR. ZEIGER: Well, my choice would be

to follow it up with the in-vivo Comet assay looking at a number of different tissues and the Pig-A mutation assay in-vivo. The in-vivo mutation assay is extensive. It takes a lot of time, and we have data from other chemicals that the tissues in which you see mutation are not necessarily the tissues in which you see tumors. So, other than the liver, you may be guessing at which tissues to sample, whereas with the Comet assay, you can sample many tissues at minimal additional cost. And it takes less time.

DR. ATRAKCHI: Right. And certainly with the Ames, we are addressing an endpoint of mutation, and the in-vivo test would need to have a similar endpoint.

UNIDENTIFIED PANELIST: We have done some work on the Pig-A assay with nitrosomethyl- and nitrosoethylurea and aromatic hydrocarbons. We have not found it more sensitive than the in-vivo mutagenesis assay. I guess it is less expensive because you do not need transgenic animals. On the other hand, with the in-vivo assay, a lot of the expense is the animals. They are very expensive, but

once you have the animal, then you can sample any organ you want, and that is not much more expensive. So, once you get around the cost of treating the animals, then the assay is not that expensive, but it is the animal part and treating the animals. But you have that with any in-vivo assay that you have to treat the animals, and you have animal costs.

DR. ATRAKCHI: Thank you.

Another question is, when extrapolating from one nitrosamine to another, should molecular weight of the nitrosamine be a factor? The default limit of 18 nanograms per day or 26.5 nanograms per day was derived for relatively low molecular weight nitrosamine. Would higher limits be appropriate for higher molecular weight nitrosamines?

Would anyone from the panel like to answer this?

DR. EISENBRAND: I may just mention drawing the attention to let us say asymmetrically substituted methyl long chain where you have quite a spacing in molecular weight. These compounds are subject to chain shortening metabolism from the end,

from the long end chain here, very similar to the fatty acid metabolism, so they end up with a common determinant finally, which is a ketocarboxylate derivate that methylates again. So, I do not think it is probably a very good idea to use the molecular weight information for evaluation in comparison to dimethylnitrosamine. We have a lot of different nitrosamines. Dimethyl, diethyl, nitrosomorpholine. Different rates but similar potency. And as I said, with the long chain ones, you finally get to a very short chain methylating analog. So, I do not think it is probably advisable.

UNIDENTIFIED PANELIST: I do not know, but you may want to on a molar basis. I think that would be a more reasonable way to make the comparison.

DR. ATRAKCHI: But it would appear that most of the nitrosamines that have been detected are of the low molecular weight nitrosamines.

UNIDENTIFIED PANELIST: Yeah, that is true.

DR. ATRAKCHI: Any other comments from any of the panelists, from my FDA colleagues on any of

the topics that we have discussed today to this point? Any other questions from the FNDs that I may have missed?

DR. KEIRE: I guess I just kind of have what may be a naþve question. My lack of familiarity with this. I guess I have heard that the larger the nitrosamine is, right. So, you have NMDA, small molecules, but then when you get to say a drug substance that may be nitrosylated, so larger, 500 molecular weight maybe because of the other steric factors, that the larger nitrosamine would be less likely to be mutagenic or carcinogenic. Is that true? Can you make that statement?

DR. HECHT: I do not think so. I would be very cautious about making a statement like that. We can compare for example dimethylnitrosamine with NMK with much higher weight and also more carcinogenic. I do not think we can make that kind of generalization.

DR. KEIRE: Okay. Thank you.

DR. GUTTENPLAN: I agree with Steve Hecht on that. Dimethylnitrosamines are potent in the

liver but dibutylnitrosamine is very potent in the urinary bladder. And it is much larger.

DR. ATRAKCHI: Well, I think maybe the tendency for such a larger molecule like what Dr. Keire was discussing is that it may come up to be negative in an Ames test based on the probable steric hindrance, or it is just going through the bacterial cell wall and will not cause the mutation. So, right there up front, the test would be negative for such larger molecules. But your response is that you will not just because it is a large molecule, it does not mean it is not carcinogenic or mutagenic.

DR. HECHT: Correct. Just look at the database. I mean look at the literature. Look at the papers that Gerhard cited. I mean there are plenty of relatively high molecular weight nitrosamines that are highly active carcinogens.

DR. GUTTENPLAN: And these might be good examples of compounds that are not mutagenic in the Ames but would be mutagenic in in-vivo assays.

DR. ZEIGER: Well, so far as I know, many of these larger molecules are mutagenic in the

Ames test. I do not think you can make that generalization.

DR. ATRAKCHI: Very good. Thank you. Any other comments from anyone on the panel? Hearing none, I think we are short of five minutes to ending the first day of the workshop. If there is nothing else, I would like to thank the panelists for your valuable discussions, and we will resume tomorrow for the second and last day of this workshop with the continuation of the questions. We will get into the chemistry and the manufacturing of nitrosamines, and we will start again at nine o'clock. Thank you very much. Thanks for everybody.

(Whereupon, the meeting concluded at 2:41 p.m.)

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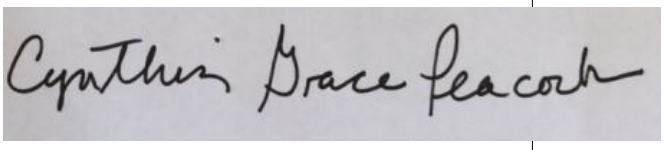

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