U.S. ENVIRONMENTAL PROTECTION AGENCY

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SYMPOSIUM ON NEW SCIENTIFIC RESEARCH RELATED TO THE HEALTH EFFECTS OF TRICHLOROETHYLENE

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Renaissance Mayflower Hotel

Grand Ballroom

1133 Connecticut Avenue, N.W.

Washington, D.C.

Thursday, February 26, 2004

8:34 a.m.

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VOLUME I

I N D E X

I N D E X (Continued)

P R O C E E D I N G S

[8:34 a.m.]

MS. SCHALK: I'd like to make some announcements, just some logistics. We are not providing any refreshments, so if you do need coffee, tea, whatever, there's a lobby bar that serves lattes, cappucinos, coffee, tea, that sort of thing. You can pick that up there. There is a restaurant list in your folder.

And each of you should have picked up a name tag and a folder. And in the folder is the agenda, the list of speakers, list of preregistered attendees, and there is a one-page fact sheet which lists the purpose of this meeting. And the purpose of the meeting is to hear some new research from the invited scientists, and we will be trying to keep on time.

Just a little on the format: each speaker has been allotted 30 minutes or 45 minutes. We're going to try to allow for a few quick questions after each presentation, but if they go over, which we hope they won't, there is a Q and A session

after each morning and afternoon session, so we will have time to address any of your questions.

And additionally, on Friday afternoon, we are going to have a one-hour panel discussion amongst the speakers, but also, we'll be able to entertain questions from the audience.

And at this time, I would like to introduce Peter Preuss, the Director of the National Center for Environmental Assessment at EPA, who will be making welcoming remarks. Peter?

DR. PREUSS: Good morning, ladies and gentlemen. I am very happy to welcome you all here. The program is quite interesting and varied. We are very fortunate to have a truly international group of speakers here with us. We have scientists from Denmark, Taiwan, Germany, the United Kingdom, so hopefully, we will get a broad perspective of some of the science that is underway now.

Just so that you understand what the symposium is about and what we are trying to do here today and tomorrow, we are in the process of revising a risk assessment for TCE. We did one a

few years ago. We brought it to the Science Advisory Board. We got a lot of comments from them, and we decided that we would need to do a significant revision.

And of course during that time, since this area is a very active one for research, we had a large number of papers dealing with the kinds of issues that we were trying to deal with. So basically, what has happened is that we have invited a large number of folks, and you can see from the program just how many there are, who have published important papers in recent times.

Again, this is not meant to be a comprehensive layout of all of the science that has been going on. That would take weeks, if not more, to accomplish that. But it is basically meant to invite some of the folks who have really published work that we think will be very important to us in the coming year as we develop this revised risk assessment.

So what that means is that the purpose here today is to hear presentations about science,

to understand what the scientists did, what their conclusions were and how they went about doing their research. The purpose is not, and I emphasize not, to discuss the implications of that research in terms of risk or to come to any consensus about what things mean.

What we are again trying to do is focus on the science so that we can understand what has been done. And as Kate has said, the way that we have set this up is that we have tried to give the speakers the maximum amount of time possible, so there is only a 5-minute period of time at the end for some clarifying questions. And I'd like to emphasize that. The questions during that 5-minute period are really meant for clarification: you showed such on slide 1 and such on slide 3, and I don't understand how they relate.

It is not meant to be what do you think of the work I have just recently completed and let me tell you about it for five minutes. So I hope we will all be able to follow that. We'll have those clarifying questions after each speaker, and then,

as Kate has said at the end of each session, morning and afternoon, we will have a half-hour period where questions can be asked and a somewhat broader-ranging discussion can be held. And hopefully, that will do the trick for us.

Now, what will happen with the results of this meeting? Essentially, we will take these as grist for our mill. As we continue to work on this risk assessment, we will incorporate what we have learned here today, and so, for us here at EPA, this is a very important symposium for us to really learn, and there are many people here from EPA, from the National Center for Environmental Assessment, and from other parts of EPA.

So in addition to welcoming the speakers and welcoming the folks from EPA, there is a third group that I would like to welcome. Probably all of you know or most of you know that TCE is a subject that is of a great deal of concern to many agencies, not just EPA. There are contaminated sites around the country that have significant levels of TCE, and of course, many of the Federal

agencies are involved in cleanup issues.

But others also have an interest as well. And so, we have set aside space for the other Federal agencies here. I think almost every Federal agency that touches on the tox issues or the EPI issues is represented here today, and I'd like to welcome those as well.

And with that, I would like to then go on to begin the symposium and introduce our first speaker. Now this is tough, because I have been practicing overnight, and I am not sure I have it yet, but our first speaker is Dr. Johnnie Hansen from the Danish Cancer Society, or as we say around NCEA, the Dansk Kraeftens Bekaemplelse. Close?

[Applause.]

DR. PREUSS: So I introduce Dr. Hansen, who will talk about cohort studies of cancer risk.

DR. HANSEN: First of all, thanks to the organizers of this interesting symposium. I am very pleased to get the invitation to be here and to present the results from our two relatively recent cohort studies.

First, however, I am going to give you a general view about our opportunities to make epidemiological studies in Denmark which is to some extent very different from your opportunities here in the United States.

It has been said that we in Denmark are a large cohort, kind of a working cohort. The reasons that we have the good opportunities to conduct epidemiological studies, not only in Denmark but in the entire Nordic countries, are at least three. We have relatively small populations. Each of the populations in the four Nordic countries is about 5 million persons. It is very well-organized, and the most important issue, I think, in this relation is that we have a central person register.

In Denmark, it was started in 1968, and it is nationwide, and it was, of course, made for administrative purposes. It covers all persons born in Denmark from 1968 and onwards and, of course, all people living in Denmark at that time. When you are born in Denmark, you receive the same

day your number. For instance, I have a daughter three years ago. We have, at my institute, online access to this register. The day after she was born, I checked if she was in the register, and she was already, although she hadn't got a name, but she had got her number.

[Laughter.]

DR. HANSEN: So what is in this register is the 10-digit CPR number, the name, the address, your job title, your place of birth, vital status, marital status, information about your spouse, your parents and your children, and it is updated as in my example on a day-to-day basis.

And the other important issue in epidemiology and cancer epidemiology is that we have nationwide cancer registries in the Nordic countries. In Denmark, this national register was founded already in 1942, and in fact, it is the oldest nationwide cancer registry in the world.

I need to present this man. It's taken 10 or 15 years ago. He is the founder of the Danish Cancer Register, Dr. Johannes Clemmesen; well,

nowadays, he's almost 100 years. Our cancer registries have been used as a model for several cancer registers in the world, and I won't go into further detail, because they are built almost in the same way, and it has been estimated that the cancer registry in Denmark is almost complete. At least 95 percent of all cancers are registered. The 5 percent that are not registered is mainly in the group of non-melanoma skin cancers.

Another important thing when you are doing occupational cancer studies is that we in Denmark have a National Pension Fund register. It was founded in 1964. And that is unique for Denmark. They don't have such a register in the other Nordic countries. The purpose of this register is that it works like a kind of bank account. All employees in Denmark are compulsory members of this system. Quarterly, the employer pays a small amount of money to this registry, and the information that is given when they pay the money is the company where the person is employed, the start of the employment, the end of the employment and the

industry code given by the Danish Statistics and, of course, the CPR number.

So in this way, when we have a person, we have the opportunity to go to this register for research purposes and reconstruct the entire history of work on a company level back dating to 1964. Since we have this CPR number, we are able to combine information from several Danish registries made either for research or, for instance, for administrative purposes.

I will concentrate on the use of these three in the next minutes. This is, of course, our pension fund, our cancer register, and we also have the access to some occupational measurements that have been performed in Denmark since 1947. Taking all of this, the entire system together where we have the labor inspection archives, the central person register, the pension fund register and the cancer register, you can see, because they all use the CPR number, it's possible to combine all of this information.

The weakest part of this is the labor

inspection archive files. The measurements have been performed since 1947. That means it was also performed before we had a CPR number. When they did the measurements, they try to write down on a form the name and the sex and the birthday of the person that was measured, but that has not been performed really good in the beginning. But anyway, these are the data we are going to use.

I need shortly to present the collaborators of these studies. It's some colleagues from the Danish Cancer Society, some colleagues from the National Institute of Occupational Health and one from the Department of Occupational Medicine, Aarhus Kommunehospital in Denmark, who did a study on the dry-cleaning workers in the 1980s and finally people from the International Epidemiology Institute here in the surroundings of Washington.

The first paper here was published about two years ago, and it is a relatively small cohort study. The hypothesis before we did the studies were in 1995, the International Agency on Research

of Cancer evaluated the epidemiological literature, and they put the most emphasis on the results found in two other Scandinavian studies on liver and biliary tract cancer and non-Hodgkins lymphomas, but after that, also, some German studies have come up on kidney cancer. They were our primary hypotheses.

From the animal assays, we know that lung cancer and testicular cancer have been found increased in animals, and also from the Finnish occupational study, there was an increased risk of cervical cancer, so it was also included in our hypotheses.

It is a cohort study, and the cohort is defined by TCE measurements. The TCE measurements were found in the archives of the Occupational Institute, where they have, for routine purposes, been trying to control the exposure levels of TCE in Denmark, and they have made some individual measurements to people on the metabolite of the urine and in the breathing zone. And altogether, we identified 803 workers that have been involved

in these routine measurements, and I need to point out that these measurements are not made for research purposes but for control purposes, so they have not been made in a systematic way.

But we have these individual measurements. We know for sure that each of these 803 persons have been exposed to TCE. From the measurement files, however, we didn't know how long time people had been exposed, but from the Pension Fund data, we were able to reconstruct their duration of employment within the same company where there have been measured.

So we were able to calculate the duration of employment within this company, although we didn't know if they had been exposed the entire time they had been employed. We followed up these people, the people in this cohort from 1 April 1968, which was the day the CPR number system was initiated, and we followed up people in the normal way until they have died; the end of the study in 1996 and so on. And as a reference group, we used the standardized general population.

A little bit about the measurement files, which were not computerized when we used them but stored in the basement of the National Institute of Occupational Health in old yellow papers. They had some information about the worker, the working conditions, the measurement and the company, but in general, they were not very complete.

So we had, in total, almost 2,400 measurements of the urine in this period from 275 companies. As a mean, about two measurements were performed for each person, and the breathing zone measurements were performed from 1974 to 1988, and we have 422.

However, for most people from this group, we were not able to identify uniquely the person, so in general, it's mostly persons that have this measurements from 1960 and onwards, although it goes back to 1947.

A little bit about these measurements. As you see, both the median and the mean are decreasing by progressing time, and the concentrations in the 1980s are much lower than in

the beginning of this period. And we see the same tendency here, although based on relatively few measurements. Graphically, it looks like this. Recognize that this scale is logarithmic.

And now, to the results of this small cohort of 803 Danish workers exposed to TCE during this period. Based on small numbers, but both the liver and biliary tract cancers increase, although not significantly, and the non-Hodgkins lymphomas, which have been found in the other Nordic epidemiological studies, have also increased relatively much.

Then, we found an increased risk of cervical cancer; the risk of lung cancer was surprisingly not increased. And when I say surprisingly, that's because this is a cohort of blue collar workers, and in Denmark and other places, it's so that particularly the blue collar

workers are smokers, and normally, in such kind of cohorts, we found an increased risk of lung cancer due to tobacco smoking, but that was not the reason here.

It was only one testicular cancer, and then, we found an increased risk of esophagus cancer, which was outside our hypotheses. Our first thought was that it may be due to a random effect found in the study. We went into some more details about this cancer. We divided it into squamous cell carcinomas and adenocarcinomas and others. We took all of these esophagus cancers diagnosed in the same period from 1980 to 1996 from men born in the same period as in our study, and we found that the normal distribution in Denmark is that the squamous cell constitutes about half of the esophagus cancers and the adenocarcinomas about 30 percent. But what we found in this study was exactly the opposite. There was a major increase in the adenocarcinomas.

Well, this is a part of the paper. We tried to stratify people during two different

indicators of exposure, but I think I regret that we put this table into the paper. I think we were pushed to do it by the reviewer of the paper. As you see here, we have only eight persons with non-Hodgkins lymphomas and six persons with esophagus cancer.

But what we found is that there is some tendency of an increased risk with increased duration of employment but don't put too much emphasis on this.

So the conclusions of our first small study was that there was no support that TCE exposure increases the risk of lung cancer, testicular cancer and kidney cancers. We found increased risk of non-Hodgkins lymphoma and cervical cancer, and we found this surprisingly high risk of esophagus cancer that had not been found in other studies before.

There are no major confounders for non-Hodgkins lymphomas. It is so

in Denmark and other places that the increase of non-Hodgkins lymphomas is particularly increased in upper-class people, but this is a study of lower-class people, so we should, in fact, expect a lower risk of non-Hodgkins lymphoma. And then, it is so that tobacco is normally associated with adenocarcinomas.

We didn't find any dose-response relationship, but this is a very small study, so maybe you shouldn't expect it at all.

Well, we are focusing on one particular chemical, TCE exposures, but these people had been exposed to several other chemicals within their employment, particularly in the metal and iron industry. But we don't know anything about other chemicals, and we do not know anything about lifestyle habits, such as tobacco smoking, alcohol

drinking and so on.

So it's a very small study, and we need results from a larger study. That was our conclusion. And that, we did.

So this is a paper published just before Christmas last year, so it is very recent. The hypotheses were almost the same as in the first study, but we also included esophagus adenocarcinomas, in order to see if we could reconstruct the results found in the first study.

The design of this study is very different from the first one. In this study, we didn't have individual measurements. Instead, we had information about the companies where they have used TCE and, we identified 347 companies with historical use of TCE. and based on the company number for these 347 companies, we were able to reconstruct the employees at these companies.

We also know, of course, that all of the employees in these companies have not, all of them, been exposed to TCE, but we had no data on who had

been exposed and who had not been exposed. So we set up some criteria. We said that they should have been exposed for at least three months in these companies; they should have been a blue collar worker; and the companies should employ less than 200 people, and I'll come back to this later.

They were followed up from 1989 or from the first date of employment that we found with the Pension Fund data. And the relative risk was calculated in the same way. So the sources of exposure information was from a nationwide produc t register survey in 1984, where companies where they used TCE were identified. There was the dry cleaning survey from 1987; the same measurement files where we now didn't use the information about a specific person, but we knew that these companies have used TCEs, and it was measured there.

And then, we got some information from the major supplier of TCE in Denmark, who helped us with identifying some companies with a major use of TCE. However, we excluded those companies with more than 200 persons, so we got 347. And the

reason for excluding those companies with a high number of employees was based on this figure, where you see that the proportion of workers exposed to an organic solvent is related to the number of employees in the company. The fewer number of employees, the higher proportion of workers, in general, exposed.

And so, we set the limit to about 200. So in our final cohort, we had these 347 companies, and altogether, more than 150,000 people had been exposed in our period from 1964 up to 1969.

We excluded the white collar people, based on their job title from the CPR register. And it was about 40,000 people. 56,000 people which we could not divide into white or blue collar were excluded also. And then, finally, we excluded those short-term workers. And so, finally, we had this cohort of about 40,000 potentially exposed workers.

And the results here, now, it's based on 2,620 observed cancers versus 2,400, and the relative risk is now significantly increased,

although it's relatively low. And to some extent, we in fact see the same pattern as in the small study, which is at least partly independent, because the cancer cases in common in the two studies are about 10 percent or something like that.

But the relative risk in these studies, and none of these are significantly increased, are, to some degree, lower than in the first study. But on the other hand, an unknown proportion of these people have in fact not been exposed.

We tried again to focus on the adenocarcinomas, and also, in this study, we found an increased risk of almost 2.0. And in this study, we also found an increased risk of lung cancer, as we also would have expected in the first study, because this is a cohort of blue collar workers compared with the general population. And the only other significant result from this study was a decreased risk of skin melanomas, which is also normally found in blue collar workers in Denmark.

I forgot to say those we presented before were men. Now, we have the females. They were combined in the first study in order to bring power. Here, we found a relatively high increased risk of liver and biliary tract cancer. We also found an increased risk of renal cell carcinomas, although not being statistically significant. And we found the same pattern for non-Hodgkins lymphomas. The number of esophagus cancers among females were very low. I won't go into details with that.

And then, we again find an increased risk of cervical cancer and lung cancer among the women. But both of these cancers are related to tobacco smoking, and we know for sure that this group of female blue collar workers smoke lots more than the general population. And we also find this expected result that they have a decreased risk of non-melanoma skin cancer.

We tried to stratify according to different exposure indicators: lag time, duration of employment, year of first employment, because we

know that from--I have shown you before that the levels of exposure were much higher before the 1970s than later. And we tried to stratify it according to the numbers of employees.

And I am not going to take you through all of the increased sites, but particularly in this, the NHL, it seems to be most consistent. And when we included lag time, we saw a tendency among the men and women to an increased risk. We found an increased tendency of increased risk when we had a longer duration of employment compared to a shorter duration of employment, and the major part of the risk was concentrated about people who had been exposed in 1970 or before.

And finally, we didn't find results that supported that if there is a causal relationship, it should be found particularly among the small companies, because a higher proportion is exposed, and higher levels have also been found here. But that was not seen.

This is renal cell carcinomas, and we see almost the same pictures as for non-Hodgkins

lymphomas shown before. There's some effect of lag time; the duration of employment seems to play a role, and the highest risk is found of those in the beginning of the period, but nothing meaningful here.

So the conclusion of the second part of the study is that there seemed to be support for the association between past levels, at least, of TCE exposure and non-Hodgkins lymphomas. Well, it's difficult to discuss confounders, because there are almost no confounders related to non-Hodgkins lymphoma, although we have as I told before the fact that it seems like the upper class people have the highest risk, but this is low-class people, so this might tend to decrease a truly increased risk of non-Hodgkins lymphoma.

The association between TCE exposure and renal carcinomas: tobacco smoking may be a confounder here; maybe also other exposures. We didn't find any dose-response indication of increased risk of female liver and biliary tract cancers; in fact, we found that increased risk for

these cancers were concentrated among short-term workers. And again, we confirmed our increased risk of esophageal adenocarcinomas, and of course, our mantra, as epidemiologists, we need further results to further confirm this.

But this is not the end of our research within this field. We, as I told you before, there are some other Nordic studies, and these are the results for non-Hodgkins lymphomas and livery cancer. And as you see, the results are very similar, in a way. So we are going to update the cohort studies from all of these countries, and that will give us almost 70,000 new person-years, and that will double the size and the statistical power of our study.

Thank you very much for your attention during these 35 slides. Thank you.

[Applause.]

DR. PREUSS: We have time for one question if anybody has something particularly urgent. I can't pick out the face, but the name is familiar. As you come up, could you please identify yourself?

We are taping this session, so we'd like to know who is speaking.

QUESTION: Lawrence Romberg from Gray Deep Corporation.

In the two studies, was there an overla p in the people? Did the people in the first study reappear in the second study?

DR. HANSEN: No, in fact, not. In the first study, where we had measurements for the individuals, they were--most of them were from larger companies that were excluded from the other study. I think it was, in general, less than 10 percent of the people who participated in both studies. So the answer is no.

DR. PREUSS: Thank you.

I have to ask, is Dr. Pesch here in the room?

[No response.]

DR. PREUSS: No. In that case, we will move ahead, and we will ask her to speak when she arrives.

The one other thing that I forgot to

mention this morning in the introductory remarks is that as you know often, there are people from the press, the media, who come to these meetings. And I believe we may have somebody from People Magazine here today, so that all of you sit up and smile and try to take the best picture you can. You never know if you are going to be on the cover.

Our next paper will be by John Cherrie, and he will talk about TCE exposure estimates. Dr. Cherrie will talk about TCE exposure estimates to determine kidney cancer risk.

Dr. Cherrie?

DR. CHERRIE: Thank you very much.

I am very pleased to be here today to speak to you all. And I would like to acknowledge the contribution of my collaborators in the work that I am going to describe. These are Hans Kromhart from the University of Utrecht in the Netherlands and Shawn Sample from the University of Aberdeen in Scotland.

I guess that the last time EPA went around and looked at the scientific evidence to do with

TCE, there was a view of the epidemiology, and as part of this, the authors highlighted the importance of good quality exposure information in coming to a robust conclusion about the differences between studies. And we would agree very much with this.

And I suppose our experience is quite different from that in Denmark in that in most other European countries, there isn't the same infrastructure for obtaining detailed information about individuals in cohort studies, and particularly in the UK and Germany and France, there is, in most epidemiological studies, a dearth of information about exposure.

In fact, in many studies, the only information that's available are anecdotal recollections of the kinds of conditions that would have existed in the past, perhaps going back 30 or 40 years in the past. So really, it can be very difficult to understand whether one study is in a comparable situation to another.

I suppose that the importance of this

comparability is drawn out most clearly in relation to the information from kidney cancer which was summarized in that review of the epidemiology. And the information, I have extracted a few of the information from the tables in that paper. The two studies that are highlighted here, one by Blair and colleagues and the other by Henschler, show the contrasting information about the risks that appear.

In the American studies by Blair, these are aircraft workers using TCE, amongst other solvents, to clean the metal parts. And some of this is done by cold-clearing, where the parts are wiped with cold TCE and others in a degreasing environment.

The study from Henschler is a very different situation. This is workers in a German cardboard factory, who were periodically cleaning the machines using TCE. The pattern of exposure is quite different; the mix of solvents is quite different, and the German authors argue that the situation that they were dealing with was likely to

have given rise to very high levels of exposure. And clearly, there are very elevated risks amongst the workers in this plant. So I guess we are going to hear a little bit more later about the German studies.

Following on from the work that Henschler published, the same group carried out a population case control study involving 58 subjects with renal cell cancer. And in this study, the controls were drawn from hospitals where the cases also originated. To assess the exposure for these cases and controls, the authors used self-reported prenarcotic symptoms as the basis for their exposure assessment, although they also had quite recently detailed information about the types of work that were undertaken and also the pattern of work in terms of the duration of employment.

And again, clearly, in these studies, there is a very high odds ratio for those who were exposed or judged to be exposed to TCE, with some suggestion that those with the lower level of exposure in the single grade on the scale that the

authors produced having slightly lower odds ratios.

But these results are quite in contrast with the other studies, for example, those that Johnnie Hansen has described. And really, we are interested to understand whether or not there was a real difference in the exposure levels between the German studies and the other work.

But as I have suggested, it is very difficult in these situations to deal with the rather sparse information that is available. And we have been developing techniques to try to estimate exposure in situations just like this, where there is only descriptive information available for the subjects' work activities, and we have developed a very simple theoretical model, which I'm going to describe in the next few slides, which tries to obtain estimates of inhalation exposure for epidemiological studies.

The basis of the model is a simple theoretical structure, where we have information obtained about the intrinsic omission, for example, the volatility of the materials being handled, the

way that the materials are handled, the types of control measures that exist, and the pattern of the exposure in terms of the time activities that are going on.

And the work itself is based around a series of mathematical formulations which, for example, in terms of emission, deal with the first three of those parameters. The models are multiplicative models, so each of these parameters is multiplied together to produce an estimate of the emission for this particular situation.

Now, the mathematical formulation perhaps hides what is, in fact, a subjective process. The scheme that we have here provides a framework but does not provide direct calculations of the exposure estimates. So it still relies on the assessor making judgments about the level of the intrinsic emission, for example, the type of handling that was going on and so on. So the subjectivity is not completely removed.

And that is really because we are dealing with a very tenuous set of information, descriptive

information about the situation. Well, I don't propose to go through each of these equations in detail, but really, just to show you that there are considerations not only of the direct work that the subjects are carrying out but also other work that is carried out within the environment, and these are all wrapped together in some sort of mathematical formulation.

Now, to be sure that the method was reliable, and we have been using this method in other epidemiological studies, we have carried out some validation studies. And these have involved descriptive information about what situations that were collected in parallel with measurements that were made.

These are not for TCE but for other materials, vapors, solids and fibrous materials. The graph itself shows along the horizontal axis the measured concentration on some relative scale and then the vertical, the assessed results from the individual assessors. The data are for three individual assessors in each of the three colors

here.

So, for example, these three points down here correspond to measurements that were made of a concentration of exposure level, I should say, of about 0.01, and you see the three assessors produce slightly differing estimates of their exposure but reasonably well-clustered. What we found in the validation studies that we've carried out is that the method tends to produce estimates which are well-correlated with measurements that are made and that there is a tendency for the assessors to produce slightly positively biased estimates of exposure.

Well, it's not a perfect technique, but then, it's a technique that's applied in a situation where there really is no clear objective measurement information available. So it provides us with a method of providing some estimate of the quantitative exposure.

So what we have done is we have applied these techniques to the two studies that I highlighted at the beginning of the talk, the

studies by Blair in the U.S. aircraft industry and those of Henschler in the German cardboard factory. Now, the real purpose of what we are trying to do in this work was to look and see what were the differences, slight differences, in the exposure level.

We have not set out to critique the studies by Henschler in terms of their epidemiological methodology or any other aspects of the studies that are relevant, purely to try to understand here, are there real differences in the exposure between the two sets of studies that might provide an explanation for the observed differences in risk.

Well, we made our estimates using the methodology, but then, we were able to go back to two other studies that were carried out in the 1950s involving trichloroethylene and to carry out the same techniques with these studies and then to use the measurements that were available from them to adjust the earlier work, so that our estimates for the Henschler and the Blair studies are

adjusted to take account of what we know in terms of the measured exposure in the other studies.

One of these studies was carried out by a colleague that I worked with many years ago, David Heckish, from the UK, where he had measured TCE concentrations and vapor degreasing environment in the mid-1950s, just about the time I was born, in fact.

And these measurements provided a very useful reference point for the adjustment of the estimated exposures. We also estimated the exposure levels for the Henschler study using a simple calculation mass balance model that would allow us to estimate the concentration in the room environment. This was because Henschler had quite detailed information about the quantities of trichloroethylene used in the processes. And in fact, these were very high; remember, this is a small, relatively small cardboard manufacturing plant, where, at its peak, used in excess of 28,000 liters of trichloroethylene each year. So on the face of it, it seems a very extreme situation.

We also attempted to use these methods in the study by Vamvakas, and remember, this is a population-based case control study, so there is less information about each of the work situations where the cases and controls were employed, so that of necessity, the methodology is much simpler in terms of its application. But nevertheless, we have tried to do that as well.

As I say, there was a very high concentration of trichloroethylene used in the peak periods for cleaning the machines. And we estimate that the long-term average concentration to TCE would be about 225 parts per million. Of course, the work wasn't carried out continuously using TCE in that the cleaning was only done once every two weeks and lasted between four and five hours on each occasion.

So this long-term average is made up of very high concentrations over those shorter periods of time. We estimate that the concentration during the cleaning could have been as high as 2,000 to 4,000 parts per million of trichloroethylene.

The minimum figure there of 70 parts per million refers to the decline--the usage of trichloroethylene declined over the years, and this figure would correspond to the lowest amount of trichloroethylene used in the cleaning process.

Now there were other activities described by Henschler in the cohort. The locksmiths and electricians were basically doing vapor degreasing activities, and we estimated their exposure to be about 100 parts per million. It's a much more even exposure regularly, day in and day out, that is described here, and the other activities are correspondingly lower still.

The studies by Blair and colleagues in the U.S. aircraft industry, well, basically these people were doing two types of work. They were either using trichloroethylene as a type of cleaner in the cold environment, wiping it onto metal parts or were using it in vapor degreasing.

The assessed exposures here, long-term average exposures, between about 50 and 140 parts per million. The 140 parts per million corresponds

to vapor degreasing where there was very little effect of controls existing. And in situations more recently, where effective controls were installed, we would assess that the exposures were likely to have been much lower, around about 10 parts per million.

The information from Vamvakas, we were able to try to estimate cumulative exposure against the three categories that the authors described, so that this is one pluses, two pluses, three pluses on their exposure scale. And on the vertical axis, we've estimated cumulative exposure in parts per million hours.

So, as I mentioned, there is a great deal more uncertainty about these data, but I guess our best estimate would be that there is very little difference in the assessed exposure using this methodology in terms of cumulative exposure than one might have expected. In other words, the three exposure categories are much more similar than would be suggested from the assessments from prenarcotic symptoms.

And the range of average exposure levels for each of these three categories does increase, from 65 to 150 parts per million. These are the exposure levels during the work activities. And in fact, this ranges over, I think the lowest average assessed exposure was around about 1 parts per million, and the highest was about 450 parts per million.

Now, we have some criticism of the techniques that were used by Vamvakas which I think are pertinent to highlight here. The first is that the interviewers who carried out the work, collected the information about exposure, were not blinded to the case control status of the subjects. And so, there is a possibility of interviewer bias in the collection of this information.

We are critical of the use of symptoms for assessing exposure, and I think the work that we've done here tends to underline that fact. Using symptoms is likely to give rise to recall bias; in other words, the cases were likely to recall symptoms than the controls.

And finally, we noted in the paper by Vamvakas that the younger cases were more likely to have been judged to be exposed than the older cases. This seems somewhat counterintuitive, because the younger cases, one would expect, have been exposed more recently. And yet, the exposure levels will have declined in industry over time. There are a number of studies which have looked at year-on-year trends of exposure in industry, and I think Johnnie Hansen's data illustrates what is a fairly general trend that as time goes on, exposure levels tend to go down year-on-year in a fairly continuous way.

So the fact that the younger cases who were likely to have been exposed more recently had the higher assessed exposure suggests to us that there was some potential there for recall bias in these assessments.

So to sum up, then, we have looked at three studies, two of the German studies that have been published and one of the American cohort studies. And we have tried to see whether there

are similarities and differences in the exposures that these individuals may have experienced.

I think that, in our opinion, there is a great deal of similarity in the long-term average exposures for all three of these studies. In the Henschler study, the long-term averages are somewhere between about 15 parts per million and 225 parts per million and the Blair studies between 10 and 140 parts per million and in Vamvakas perhaps between 1 and 400 parts per million.

That is not to say that the pattern of exposure is the same. In fact, what we have seen is that the pattern is quite different between the studies; that in the Henschler studies, there were shorter periods of activity which gave rise to these long-term average exposures, and the peaks there may have been up to 200 parts per million for up to four or five hours on a regular but infrequent basis throughout the period.

These peaks would have declined as time went on, and perhaps later in the production history of the plant, they would have perhaps been

as high as 200 parts per million rather than the 2,000. The peaks in the Blair study from the information that they provided in the paper were perhaps as high as 600 parts per million, and in Vamvakas, they could have been up as high as 800 parts per million, in our opinion.

In fact, when we compared the studies, we felt that there was considerable similarity in the pattern of exposure and the type of activities in the studies by Blair and those by Vamvakas. Both involved mixtures of solvents; both involved use of the trichloroethylene in cold cleaning and also in vapor degreasing situations. And we think that the similarities there are much greater than perhaps with the Henschler data.

Well, thank you very much.

[Applause.]

DR. PREUSS: Thank you, Dr. Cherrie.

Are there any questions? Again, please identify yourself.

QUESTION: Jonathan Borak, Yale University. Sir, a couple of points I'd love

clarification. I may have misheard. One, it's my understanding that in the Vamvakas study, the controls were actually motor vehicle accident victims taken from a different hospital, not taken from the same hospital. And I believe that the description both in Henschler and in the followup of the Bruening and Bolt review indicates, among other things, one, that the workers often could not work through an entire eight-hour day as a consequence of solvent effects, and I believe that Bruening and Bolt say that they could not reconstruct the actual exposure scenario because in a contemporary workplace, it could not be done, the exposures had been so high.

Given those statements, I'm surprised by your findings.

DR. CHERRIE: I would say I think you're exactly correct in terms of the controls for the Vamvakas study. Yes, I agree with you.

I think we are seeing that the exposure levels were very high in the Henschler study but only for the periods of time that the work was

going on and that the cleaning activities were not a continuous activity but were intermittent. And so, there would have been periods of very high, up to 4,000 parts per million, we think it could be at its maximum but that on a long-term average, the exposures would have been much lower, because they would have been compensated for by periods when TCE was not being used as a cleaning agent.

QUESTION: Bill Brock, Environment International.

I'm a little confused with your comparison between the Blair and Henschler study, particularly as it applies to vapor degreasing.

DR. CHERRIE: Sorry, I can't hear you very clearly.

QUESTION: Pardon me?

DR. CHERRIE: I can't hear you very clearly.

QUESTION: Well, then, I'll yell.

I'm a little confused with your Blair and Henschler comparison as it applies to vapor degreasing. In the Blair results, you suggested

that the higher levels were 140 ppm with little or no control, I think you said.

DR. CHERRIE: Yes, with very little control on vapor degreasers.

QUESTION: Sorry, I didn't hear you.

DR. CHERRIE: With very little control.

QUESTION: Okay; thanks.

Yet, in the Henschler study, I'm not sure I heard whether there were controls for those levels. You can answer that one. Secondly, do you know, since a lot of your calculations have a lot of subjective measurements, you suggest that maybe the vapor degreasing operations were quite different in terms of whether they were closed-top, open top. Can you comment on that?

DR. CHERRIE: Well, the situation in the Henschler studies was that there were no controls used when the material was used to clean the machines down. These were, as I understand, cardboard manufacturing machines which were periodically cleaned by the operators so the that TCE was used there to wipe down the machines. And

the description suggests that there was perhaps two to three liters of TCE being used each minute during this cleaning operation, so that there were very large quantities of TCE being used in a relatively small plant.

DR. PREUSS: Any other questions?

Okay; last one, then.

QUESTION: Paul Deergard from Halogenated Solvents Industry Alliance.

It's probably worthwhile pointing out that the Henschler cases were not all workers exposed during the cleaning of the machines. In fact, I don't have the numbers in my mind, but I believe it was a minority of the cases who were actually involved in the very high peak level exposures, that the majority of the cases actually were the instrument makers exposed in a pattern you would expect to be much more like the Blair type.

DR. PREUSS: Thank you.

And we will move on to our third paper of the morning, introducing Dr. Beate Pesch from the Institute of Ruhr Universitaet, Bochum, and she

will talk about the results of the German studies, particularly renal cell cancer risk and occupational exposure to TCE.

DR. PESCH: Good morning. I have to apologize for my delay, because I suffer from flu, and I shall report about the German studies conducted on the renal cell cancer risk of exposure to TCE.

I'm from an institute of occupational medicine in Bochum, and in Germany, we classified TCE other than other countries already as carcinogenic to humans based on effects on the tubular system of the human kidney, whereas, the International Agency for Research in Cancer only classifies TCE as probably carcinogenic to humans.

In Germany, there were several studies conducted on TCE-related renal cell cancer risk, three studies in the Arnsberg region, more or less, one group of scientists, and another group of German epidemiologists conducted the so-called multicenter urothelial and renal cell cancer study in five regions of Germany, and further but not

shown here, Germany contributed to the international renal cell cancer study. And a few cases from the Berlin region were implemented in this international renal cell cancer study.

So I would like to quote from the IARC summary evaluation: a study of German workers exposed to trichloroethylene revealed five cases of renal cancer, whereas no case was found in an unexposed comparison group. The study may, however, have been initiated after the observation of a cluster.

So in total, three studies were conducted in the Arnsberg region, which is a district in North Rhein-Westphalia, more a rural district, not as highly industrialized, as mentioned in the paper. It is a skiing area, like Winterberg. And this region is not elevated in kidney cancer mortality. It is German average in mortality for kidney cancer.

And these three studies are different by study design. The first study of Henschler was a cohort, I would say a 'make-believe cohort' in a

cluster, because a true cohort study would have reported all cases of cancer, not only renal cell cancers or kidney cancers. It was initiated after the observation of a company doctor, likely after introducing sonography in the company because of three out of five cases' year of diagnosis was around 1990, so maybe it was a lead time bias, since cases were detected earlier, due to the sonograms.

And so, Vamvakas' study was not a population-based; it was a hospital-based case control study with only 58 renal cell cancer cases; 84 controls, completely unmatched, with an 11-year age difference in average between cases and controls. As already mentioned, from different hospitals, the controls were recruited from different hospitals but not from the hospital where the cases came from.

So the next study, the Bruening study, selected 134 cases and more controls, 401 controls, also hospital-based, but to recruit elderly controls, nursing homes were used. It was also not population based, and therefore, this has to be

considered as a methodological shortcoming.

Exposure assessment was already mentioned. In the Vamvakas and Bruening studies, a questionnaire was used where the cases and controls were asked for exposure, TCE exposure, so it was a self-assessed exposure assessment. In the Vamvakas study, experts were included to use also compensation reports of cases about details of the work activities, and so, they reconstructed exposure scenarios.

In the Bruening study, in addition, the job titles of the cases and controls were used for employing a British job-exposure matrix. They also used a database compiled by the Finnish Institute of Occupational Health, which very roughly estimates the probability of being exposed to TCE by industries. The risk estimates in all three studies are much higher than reported in the literature.

The Henschler study resulted in a standardized incidence ratio (SIR) of 8 based on these five cases where expected cases were calculated from the Danish Cancer Registry. And based on the

local mortality of the federal state, so the mortality was not significantly increased based on two deaths from renal cancer, but the mortality was not based on death certificates but just on hospital records.

In the Vamvakas study, the risk for overexposure to TCE based on self-assessed exposure was around 9, which is much higher than in the literature, and for long-term exposure, but I could not find in the article what means long-term exposure, and also, I could not find a table which shows the exposed cases and controls, was reported with around 11.

In the Bruening study, ever TCE exposure also self-assessed was 2.5, and for more than 20 years' exposure, it was around 2.7, not significantly elevated but based on the small numbers. For the Bruening study, these two additional exposure assessment methods were applied.

So, in the job exposure matrix of Pannett, this British matrix, there was a rating for degreasing agents. And this was applied to the job

titles of the cases and controls. Exposure to degreasing agents was not shown to be a significant factor. And on the other hand, if we used this very rough estimate for any working in industries with potential exposure to TCE or PER, we came to an odds ratio of 1.8, but if you look at the numbers of exposed cases and controls, you see that a large faction of cases and controls would be considered as being potentially exposed.

In addition to the epidemiological data, a subgroup of cases from the Vamvakas study with TCE exposure and without TCE exposure was sequenced for mutations in the VHL gene, and several of the cases, 9 out of 17 with TCE exposure, have been shown to be carriers of a so-called hot spot mutation, whereas among non exposed cases, not such a mutation could be shown.

The other study was a so-called multi-center urothelial and renal cell cancer study, and this study was conducted in the early 1990s in regions of West Germany, which was Berlin, Bremen and Leverkusen and in East Germany in Halle and

Jena; in total, 935 cases were enrolled and more than 4,000 population controls.

For exposure assessment, we used job titles and job tasks, respectively job activities. For assessment of TCE exposure, duration of working in special job titles or special job tasks like metal degreasing was used. In addition, we applied also this Pannett job exposure matrix and another German job exposure matrix. And for the job activities, we developed a job task exposure matrix. And based on these instruments or exposure assessment methods, we calculated TCE-related risk estimates, and the exposure index was used to define medium high and substantial exposure, with cutoffs based on percentiles of the distribution of this index among exposed controls.

And you see that the risk estimates for TCE exposure with these methods were just around 1.0, 1.1, 1.2, and there was no dose-response relation and not a clearly-shown increase in risk. We also used the job activity ‘metal degreasing’ in men for an estimate if TCE exposure could be

associated with a renal cell cancer risk. In the Bruening study, ever working in the job activity, which was metal degreasing, was associated with a significant increase of risk, and in the larger multi-center urothelial and renal cell cancer study, even very long exposure in such a job activity was not associated with an increase of risk.

So, the conclusions from the German studies are that the Arnsberg studies show high risk estimates, but the methodological shortcomings have to be considered, and the other larger study, the MURC study, could not show clear evidence for a TCE-related renal cell cancer risk.

Thank you.

[Applause.]

DR. PREUSS: Any questions?

[No response.]

DR. PREUSS: Okay; thank you very much.

Why don't we take our morning break, then, and why don't we ask everybody to come back--it's now 5 to 10:00. Why don't we ask everybody to come

back at 10 after, take a 15 minute break, and then, we'll be able to start promptly with our next session.

Thank you very much.

[Recess.]

DR. PREUSS: If everyone could take their seats, I would like to introduce the next speaker. We are changing directions slightly with these next three papers, and the first one will be presented by Dr. James Lacey of the National Cancer Institute, and he is going to be talking about scleroderma and solvent exposure in women.

DR. LACEY: Thank you. Good morning, everyone. The title slide is correct. I am at the National Cancer Institute, but this morning, I will be talking about scleroderma and rheumatology.

I've been at NCI since 1998, but these data are based on work that was completed before then, when I was at the University of Michigan.

To begin, systemic sclerosis is one of the connective tissue diseases that's considered autoimmune. It's characterized by thick and

tightening skin, especially on the fingers, forearms and torso. You see analogous changes in internal organs, particularly the lungs and the esophagus.

The major pathogenic events involved in systemic sclerosis are scleroderma, vascular changes, changes in the endothelial cytoarchitecture, disregulated immune system function and especially increased collagen synthesis and deposition. That accounts for the tightening and thickening skin.

Etiology is essentially unknown. It is recognized as extraordinarily complex, and so far, none of the hypotheses is considered unifying or to singularly capture all aspects. It's clear, though, based on those clinical features that I mentioned some key cell types are involved, particularly fiberblasts, endothelial cells and obviously the immune system.

The epidemiology of this disease clearly indicates that it is a rare condition. In the U.S., the annual incidence rate is 20 per million

per year. U.S. prevalence, at any time in the States, there are about 240 cases per million persons. Those two statistics combine men and women. Both are higher in women than in men; in fact, the incidence is about 39 per million per year in women and about 9 per year per million in U.S. men.

Because of its rarity, representative studies of this condition are considered difficult. Case definition, as I'll show you, is an issue. As a result, the literature includes many case reports and hospital-based series. There are few what we would call rigorous population-based epidemiological studies.

It was against that backdrop that the University of Michigan decided to conduct one of those studies, a case-control study in the two states of Michigan and Ohio. The objective was to get systematic investigation of potential risk factors for scleroderma, including environmental factors, medical conditions and some other factors, using a population-based case control design. One

of the motivations for the study was the potential association between scleroderma and silicone breast implants.

Cases for the study were women at least age 18 at the time of diagnosis, and diagnosis covered 12 years, 1980 through the end of 1991. In Ohio, the ascertainment period was extended by one year.

We identified cases from four potentially overlapping sources: first, because coinvestigators were on staff at the University of Michigan and Wayne State Universities, a search of those affiliated hospitals identified incident cases of systemic sclerosis. We also used a national hospital discharge code database, Health Care Industry Analysts, based in Ann Arbor, Michigan, which could identify all discharge codes for scleroderma.

We also mailed letters to all Michigan and Ohio rheumatologists, asking them to identify their patients and welcome them to participate. Included in that mailing were other relevant specialists,

like dermatologists who would have been likely to treat patients with scleroderma. And at the time, the Scleroderma Foundation was known as the United Scleroderma Foundation. It's a support group for patients' families, and we used mailings of the Southeast Michigan chapter of that organization to help identify potential patients. It's estimated that in the two states, we identified between 75 percent and 80 percent of all eligible women.

Here's how we defined cases: based on medical record review, we use a 1980 American College of Rheumatology classification criteria, where any patient with a major criterion, proximal scleroderma, or two or more minor criteria, sclerodactyly, pitting scars or pulmonary fibrosis.

We also considered a case group of what we called probable scleroderma. These were patients who had signs and symptoms characteristic of scleroderma, and this is usually referred to in the rheumatology literature as CREST, as a combination of these features. As I said, especially in Michigan, where we had a little better estimates

due to the affiliation of those hospitals, we think we identified 80 percent of all incident cases.

Controls were selected through random digit dialing, frequency matched within state on age and race. Response was a little bit higher in Michigan than in Ohio but good in both states, and the control to case ratio was about 3 to 1.

The telephone interviews were conducted by the University of Michigan's Institute for Social Research between 1992 and February 1996. It lasted approximately 30 minutes and covered a range of exposures: demographics, family history of diseases, occupations and hobbies, which I'll show you a lot more about, reproductive history and other health factors.

The exposure assessment was, I think, one of the strengths of this study. We used two approaches to try to get solvent exposure. One was to ask about occupations and hobbies that have a high probability of exposure to solvents. We considered exposure to be at work for at least once a week for three months or more, and we asked about

ever work with solvents within those occupations and hobbies.

We also asked the cases and controls about work with the individual solvents, again using a baseline of at least once a week for three months or more to be considered exposed.

Here are a list of those 16 jobs or hobbies. I do want to go through those in a little detail: dry cleaning, chemical or dye manufacturing, petroleum refining, vinyl chloride manufacturing, plastics industry, rubber product manufacturing, painting or paint manufacturing, furniture refinishing, hair dressing, work in a medical or diagnostic or pathology laboratory, professional cleaning or maintenance, film developing or publishing, perfume, cosmetic or drug manufacturing, fiberglass industry, leather tanning or shoe manufacturing, and arts or crafts. Obviously, TCE isn't necessarily implicated in all of these, but we were interested in some other exposures as well.

So any woman who reported that they had

worked in any of those jobs or hobbies for the requisite time was then asked open-ended questions about the years in which she worked, her job title, the specific tasks, the name of the place at which she worked and the type of main industry or business.

In addition, each woman was asked within those jobs or hobbies whether she ever worked with nine individual solvents or categories of solvents. And women who reported solvent use within those jobs or hobbies were then asked the years in which they first and last used the solvents, whether they worked directly or near the use of the solvents, and whether they wore protective clothing during that work.

Here's the list of the nine on the left, again, TCE is up at the top. We also asked about--we asked whether there were any other solvents that were used during those jobs or hobbies, and then, for all women, including the ones who reported no job or hobby exposure, we asked whether they had worked individually with

these solvents.

So, expert review was used to review each of the reported exposures. Dr. David Gerbrandt and I sat and reviewed each of those open-ended responses. We reviewed them blind to case control status, and before reviewing them, we assembled reference materials to look at the typical processes used in these activities, the types of solvents used in those tasks. We were able to assemble some data on exposure levels associated with individual tasks and importantly to get some documentation of the historical time periods at which those solvents were used.

We considered exposures to be confirmed when the solvent was commercially or industrially available, when it was reported used, and there was some documentation that the reported solvent was likely to be used in the reported task or hobby. Also, exposure had to be of nontrivial frequency, intensity or duration. And when we saw a report that we considered implausible or trivial level, we considered that not confirmed.

Statistical analysis was pretty standard for case control studies, except we did adjust for year of birth and attained age to address both cohort effects and opportunity for exposure. And we considered the solvent exposures only if they occurred before the case's age of diagnosis.

So controls were compared to women born in the same year of the same age, and if a control had been exposed to a solvent after the year in which the index case was diagnosed, that wasn't used in the estimating of risk associations.

Here's some demographics about the study population: 660 cases, over 2,000 controls. At interview, cases were a little bit older than controls by about 5 years, and there was an average of about 7 years between diagnosis and interview for the cases. Most of the participants were white. Current smoking was less common in cases. We think this is probably as a result of the onset of disease. Obviously, with pulmonary conditions, women diagnosed are much less likely to smoke. And SES was generally high in this group.

Here are the data on TCE: a total of 8 cases and 15 controls reported any exposure to TCE. The odds ratio for that exposure was 2.0, and here's the 95 percent confidence interval. In both cases and controls, we considered only half of the reported exposures to be confirmed. And when we analyzed just the confirmed, expert-reviewed exposures in 4 cases and 8 controls, the odds ratio was about the same but just slightly lower, 1.9, with a wide confidence interval.

Here are data for TCA exposure, trichloroethane. Similar numbers, similar levels of confirmation, although the odds ratios are closer to unity, again, with wide confidence intervals. Data for perc essentially tell the same story, with odds ratios right around the middle.

Of that long list of jobs and hobbies, these are the three that we thought were most likely to involve potential exposure to TCE: professional cleaning or maintenance was much more commonly reported, an odds ratio of 1.8 with a confidence interval that excluded 1. Work in the

plastics industry or rubber product manufacturing was less common, and odds ratios were lower.

I do want to note, though, we did not set out to expert review the jobs and hobbies per se. Instead, the expert review was focused on solvent exposure within those jobs and hobbies.

So, summary: TCE exposure was positively but not statistically significantly associated with scleroderma. There was a low frequency of exposure in both cases and controls, and half of those reported solvent exposures were not confirmed. However, when we limited analysis to just the confirmed exposures, that positive but nonsignificant association remained. And so, we thought that overreporting alone didn't appear to account solely for that elevated odds ratio.

I do want to address the anti-scl-70 antibodies. A case-control study from Paul Nitert down at the University of South Carolina reported that there was a positive association between TCE and scleroderma in men who tested positive for this particular autoantibody. They hypothesize that

solvents might bind topoisomerase and trigger an autoimmune response.

Based on those medical records that we reviewed for all cases, we did extract some clinical and chart information; anti-scl-70 antibody status was known for 250 of the 660 scleroderma cases. But none of those eight cases who reported exposure to TCE had documentation of positive antibodies for anti-scl-70.

Within the study, we included a separate case group of what's called undifferentiated connective tissue disease or UCTD. This was motivated by the fact that a lot of rheumatological conditions take many years to develop, and many patients present with signs and symptoms that suggest a rheumatologic disease but that don't meet diagnostic criteria.

So we assembled a case group of patients with signs and symptoms but who didn't meet current classification criteria. It was a smaller group, 205 total cases. Using the same approach with the same control group, TCE exposure was only reported

by one UCTD case. Interestingly, that exposure was confirmed but the odds ratio essentially showed no association, although any estimate of risk based on one exposed case is tenuous.

So, strengths of this study: I think the large study population from those two states is a real strength of this study. It was a very reasonable attempt to get almost all cases within those two areas. Participation was high, especially for population-based case control studies. We collected extensive data on not only these exposures but on other risk factors, and I think the expert review aspect of our study was certainly a strength.

Some of the limitations: there was low frequency of reported exposure. Some of this, obviously, is a function of the source population. We were looking only at women. And as I mentioned, our expert review was only applied to reported exposure. We had no information and didn't attempt to infer any information about exposures that weren't reported.

Selection and information biases are a function of or are a potential concern in any case control study. I think our high levels of participation and our standardized interview did as much as we could to mitigate those potential biases, and as I said, these results are based on women only.

So a conclusion: I think these data provide some suggestive evidence of an association between TCE exposure and scleroderma in women. But I would not consider these data conclusive at this point. I think our study reminds us that exposure assessment is critical, and to identify and verify specific exposures in populations that are large enough to get valid risk assessments is a real challenge. And as we heard in some of the earlier talks this morning, we didn't address bystander exposures, but those are obviously an important component of potential TCE exposure.

I do want to recognize the research team: Dr. David Schottenfeld was the study PI. Dr. David Garabrant was the first author of the scleroderma

paper, which appeared last year. This is the paper on undifferentiated connective tissue disease. This is a list of coinvestigators.

Our funding came from a combination of NIH grants and primarily support from the Dow Corning Corporation. Dr. Garabrant also received funding from the Halogenated Solvents Industry Alliance during the latter half of the study and during some data analysis. So I present that to you for purposes of full disclosure.

And again, this was worked on all at the University of Michigan, not when I was at the National Cancer Institute.

So, I would welcome any questions.

[Applause.]

DR. PREUSS: Yes, sir.

QUESTION: Jay Pandhi from the Medical University in Charleston.

Most of the patients were diffused or limited?

DR. LACEY: Most were diffuse.

QUESTION: What was the percentage?

DR. LACEY: I don't recall off hand. I've got the paper--

QUESTION: Second question: what method did you use to measure the anti-topo 1, and did you have a chance to look at any other scleroderma-specific autoantibodies?

DR. LACEY: We based those data entirely on information that was documented within the retrieved medical records. Obviously, that would be one area that a future study would want to look at, to get systematic collection of all those data.

QUESTION: Hi, Henry Shure from the USEPA.

I was just wondering if you could clarify whether or not the controls who are in this study were possibly subject to other exposures to the same agent that may not have been considered part of the workplace, for example, environmental exposure, water, vapors, that kind of thing.

DR. LACEY: I'm not sure I fully understand the question.

QUESTION: I was just hoping to ask you if you could make a standard in regards to whether or

not the controls, that is, the cases that occurred within the control population, may have also been exposed to this agent under consideration here, TCE, from an exposure which was not part of your investigation.

DR. LACEY: Two aspects: one, to clarify, no cases occurred among the control population.

QUESTION: I see.

DR. LACEY: So those were separate populations.

If there's some confusion about the analysis I presented, I could go into that if you like. But the other aspect of TCE exposure through other unreported events, it's possible. We obviously set out to make that list of jobs and hobbies and ask about solvents to be as inclusive as possible. There is the potential that we missed exposures, although we tended to see in looking at those details of reported use that most participants were eager to report what they thought was any potential exposure.

And by asking also about other solvents or

did you work with these solvents in any other setting, I think we did as well as we could have to capture those, but we can't rule out the possibility.

QUESTION: Thank you.

DR. PREUSS: Thank you very much.

We now have two papers dealing with exposure through water. The first of these will be presented by Dr. Wang of the National Taiwan University, and he's going to talk about increased mortality odds ratio of male liver cancer in a community contaminated by chlorinated hydrocarbons in groundwater.

Dr. Wang?

DR. WANG: First, I would like to express my appreciation to the organizing committee and also to my colleagues, because this study was a team work. Actually--we tried to conduct measurements on the underground water. We measured about 49 wells. So it is a tremendous work to my team, and it is not purely out of several persons, several people's work.

Today, I am going to share with you that we have found increased mortality odds ratio of male liver cancer in a community contaminated by chlorinated hydrocarbons in groundwater. This plant is--I call it “R” factory. Actually, it is originally owned by an American but later purchased by other companies. And this one is a very famous one, RCA. I believe that all of you have heard this name. This former electronics factory was in operation from 1970 to 1992.

In 1994, because of some anecdotal reports, the Taiwan EPA conducted a study and found that there was hazardous waste dumped in the back yard of this factory, and there was soil and groundwater contamination. And this is the picture of factory--if we from the entrance just take a look it will be something like this.

And in 1970s and 1980s, chlorinated solvents were used very commonly as degreasers. In Taiwan, TCE traditionally was used very frequently until 1974, when it was banned because of an outbreak of hepatotoxicity among women workers who

used it to clean their benches. Later on, the tetrachloroethylene replaced it and also the 1-1-1 trichloroethane, and all of them are dense, non-aqueous phase liquids.

Actually, I took this chart from one of the EPA documents, which indicates that under anaerobic reductive dechlorination reaction under the ground, PCE can produce TCE, et cetera, and all of them can produce vinyl chloride, a cercinogen. And therefore, when we conducted our measurements, we tried to evaluate 19 different chemicals, including all of them.

Because of the documentation and the legal problems, the RCA factory was purchased by GE (General Electric) and later purchased by Thompson. And they tried for half a year spending quite a number of--quite a big amount of money trying to dig a pit and trying to wash the soil, underground soil and the water. The area that covered this pit was about 1,000 square meters wide. The depth was about 8 to 10 meters and reached the first aquifer.

And I'm just trying to show you from different angles. Right now, it's all covered up.

This is a historical picture. Our study was to investigate the association between if there is any increased cancer mortality risk after exposure to chlorinated hydrocarbons in groundwater. And we tried to compare the downstream community with the upstream community.

Our study design is using a case control study design or mortality odds ratio design. We collected death certificates from two adjacent villages. Probably, I should say that upstream villages up here, and they have already had the intact water supply, but we still could identify three wells. So we have three wells identified. And these downstream villages, before 1994, they still used well water. So we could identify a lot of wells.

And from a door-to-door survey, we tried to take samples from all of these wells. In total, 69 samples were taken. And there are three wells over here, and this is the groundwater flow direction. It was estimated that the grou ndwater flow, the flow rate was about 0.24 meters per day

to the north and northeast, this direction. And these were the PCE levels that we measured.

In fact, out of these 19 chemicals that we suspected, we could identify vinyl chloride, tetrachloroethylene and trichloroethylene, and you will see that the median was about 28 micrograms per liter and about two-thirds of them above the maximum concentration level. And we also identified TCA and also some others.

The well water on the upstream villages that we can identify, there were only two wells, and all of them were within safe levels. So we tried to classify the exposed people who lived on the downstream and unexposed people who lived in upstream villages. And then, we tried to use death certificate data plus the national cancer registry to identify all of the cancers.

Okay; these are TCE, and this is --the picture was taken from the air, so you can see that this is the provincial route number one, and there is a tertiary health care center over here. And here is the upstream; here is the downstream. So

there would be a good comparability for health service accessibility.

But as you can see, the red spots, actually, the pit was dug just adjacent to these red spots. The red spots were wells outside of the factory, and the pits that I just showed you were inside the factory. So very high concentration over here. And the direction of the water is approximately on this side. And these are TCE, basically the same.

Our study design was a mortality odds ratio. Briefly, it is--actually, we took every kind of cancer as the disease of interest. And then, we tried to deliberately take controls or control diseases or reference diseases. From case control study design concepts, control should be chosen unrelated to the exposure, which means that if it is a disease, then, the incidence rate or the mortality rate of the reference disease among exposed and non-exposed were the same (based on this assumption).

So we tried and think that probably,

cardiovascular and cerebrovascular disease may be the most suitable one, because they are unrelated to exposure to chlorinated hydrocarbons, other than arrhythmia-related deaths. So we excluded arrhythmia-related deaths and tried, in order to see, or to determine if this C over D is stable, we used all noncancer diseases as an alternative choice for controls and C to D odds ratio, whether these odds ratios are stable or not.

Then, mortality odds ratio for various kinds of cancers were stratified, were estimated and after stratified by age, gender and calendar period. And conceptually, we used the first period as a self comparison as also the nonexposed period, allowing for about 10 years as the induction time. And then, we tried to compare exposed and non-exposed and tried to compare this period with the baseline period, and then, we tested for trends, okay, for three periods.

This period is used as a baseline, and age, there were four strata. And then, we computed the Mantel-Haenszel summary odds ratio and used a multiple logistic regression model.

The first period, actually, was stable. The first period did not show any increase of odds ratio for the exposed and nonexposed. And then, it increased for liver cancer, further increased a little bit for two consecutive time periods. When we used all noncancer diseases as the reference causes or the controls, they still showed the same thing.

Female residents did not show the same trend, except that there is non-significant, but there is some increase over here. When we compare with the--okay, we adjusted the mortality ratio for cancer in men by residential area and time period, which means that we used the period 1966 to 1979. Later on, we decided that full operation is from 1970 to 1979. Full operation began in 1970, and they consumed approximately 3,000 gallons of perchloroethylene per year.

So we tried to use this as a baseline, and we found that all cancer increased and also liver cancer increased and also for both periods. There was also an increase in lung cancer, but there is

no such trend later on.

So, we found a significant period effect in the downstream village, and the neighboring alternatives communities with the same, with similar socioeconomic characteristics also show, if we use that as a nonexposed group, also showed the same thing. This upstream community, actually, they were less white collar workers; the exposed workers, they have a higher percentage, a little bit higher percentage of education, so it seems to me that potential exposure of these residents to other hepatocarcinogens or occupational hepatocarcinogens were unlikely.

However, we have a limitation, because we did not measure individual exposure levels. We only used something like an ecological study, an ecological assessment of the exposure dose. And not all potential confounders could be controlled, because we used death certificate data. We have other supporting evidence from a health risk assessment. I'm going to show you some of the data that we found.

And we have another study, which we conducted a mixture of using ICR mice to be exposed to this mixture of halogenated hydrocarbons, almost the same concentration as we came across at RCA site; the high concentration, which is very similar to the actual condition, and we found that liver adenoma or hepatocellular neoplasm in males increased, and mammary adenocarcinoma in female mice increased. It was also published.

These are our risk estimates. We tried to obtain cancer slopes from different databases. I'm sure that probably a lot of them were coming from our audience. Okay; and then we got the cancer risk. And these are risk estimates coming from PCE or TCE to about 10 to the -4. And we also have, as I said, that we found that vinyl chloride in underground water. But the cancer risk order was 10 to the -6.

About the possible mechanisms, we think that chemical hepatocarcinogens may cause a synergistic effect on hepatitis B carriers, especially if people were exposed to alcohol or aflatoxins. And that's the reason I try to explain

why we only found increased risk among the males instead of both men and women.

There is another possibility, that the mixture of chlorinated hydrocarbons, exposure to mixtures might increase the risk, but we are not so sure. We hope that in the future, that we should proactively prevent any persistent DNAPL pollution.

So my conclusions were as follows: we found a significant association between residence at a groundwater contaminated community and male liver cancer. But because we do not have any individual exposure data on groundwater exposure, it still cannot be generalized too much. We do try to collect additional information on potential confounders such as the prevalence rate of hepatitis B in these two different villages, and they could not explain such an association.

There was no arsenic in the underground water over here, and the average consumption in the 1970s of alcohol and cigarettes per capita, in other words, I am also using ecological data, were very similar in the upstream and the downstream

area. And we found that biologically, they are plausible from other evidence.

Thank you for your attention.

[Applause.]

DR. PREUSS: Questions? Please? Don't forget to identify yourself.

QUESTION: My name is Dick Bull from MoBull Consulting.

I am curious about how the water is distributed in those communities, because you have very spotty contamination of the downstream wells. And is it brought into a municipal system or do individuals draw water from those wells?

DR. WANG: According to our understanding, they dump directly all these DNAPLs into the ground. This was originally. Did I answer your question?

QUESTION: How is the water distributed to the people in the community?

DR. WANG: Okay.

QUESTION: Because you have very heterogeneous contamination of the wells. Are they

brought into a municipal system and then distributed, or do individuals get their water from individual wells?

DR. WANG: Thank you. Before 1994, everybody just, you know, they dug their wells, and they used water from their wells on the downstream area. The upstream area where already we have tap water already. After 1994, tap water or clean water was sent over here, and now, tap water was installed.

QUESTION: Bill Scott, Dow Chemical. You mentioned--can you clarify, you mentioned something about hepatitis B being a potential synergistic confounder. But in relationship to just males? I was a little confused by that.

DR. WANG: All right; hepatitis B, we have a very high prevalence rate of hepatitis B in Taiwan, approximately 15 percent. Beginning in 1984, 1985, all newborns in Taiwan were immunized, or were conducted vaccination. So it will no longer be a problem in the future. But currently, our prevalence rate was about 15 percent and not so

much different between males and females.

However, the hepatocellular carcinoma, the incidence rate of hepatocellular carcinoma was about two to four times in men as compared with women. This is something that we have been conducting our study and tried to explain.

QUESTION: Arthur Chainwood, Exxon Mobil. Do you have any information as to how many people in the community worked at the factory?

DR. WANG: We have excluded people who--actually only three cancers, okay? Three cancers. In total, we have about--let me take a look. I cannot remember all of the actual words. 266 cancer deaths, but only 3 occupational cancers were from the local community, local residents.

During the 1970s and the 1980s, usually, these factories, they hire a bus to transport workers, and also, they built their own dormitory inside the factory. So most of them were from outside. Actually, in our study, we excluded these people who were workers inside this factory.

DR. PREUSS: Thank you very much, Dr.

Wang.

Our final speaker of this morning is Dr. James Burch from Colorado State University, and he will talk about neurobehavioral effects of exposure to TCE through a municipal water supply.

DR. BURCH: Good morning, everyone, and thank you for inviting me here today.

I'm going to be talking about a series of studies and our most recent analysis that involves a contaminated site in Colorado. I put the citation up for this just to remind you that we have published this recently. I did bring a couple of reprints for any of you who are interested in reading the details of this study. Please see me afterwards, and I'd be happy to give you a copy of that.

I'd also like to acknowledge the co-authors of this investigation. Dr. John Reif is an epidemiologist in the Department of Environmental and Radiological Health Sciences at Colorado State University. He's the senior author on this paper. Dr. Jay Nuckols has a joint appointment in our

department and at the National Cancer Institute here. He has expertise in geographic information systems and exposure assessment for epidemiologic investigations. Linda Metzger is an epidemiologist at the state health department in Colorado helped collect a lot of the data. David Ellington, who is a water resources engineer, helped with the modeling effort, and Dr. Kent Anger is a neurobehavioral specialist at the Oregon Health Sciences University.

I'd also like to acknowledge ATSDR, the Agency for Toxic Substances and Disease Registry, who provided the funding for this investigation.

As a little bit of background to introduce this, we have already heard that TCE is used widely as a solvent in industrial processes, as a degreasing agent; approximately close to half a million workers in the U.S. are thought to be exposed to TCE. And as a result of these industrial uses, TCE is also considered the most ubiquitous contaminant found at national priority list hazardous waste sites throughout the

United States.

There is an extensive literature on neurotoxicity associated with TCE and other solvents, as I am sure many of you are aware. And so, neurobehavioral abnormalities can be predicted from laboratory animal investigations as well as the limited human studies of acute and chronic exposures that have been performed.

As a little bit of background for this investigation, trichloroethylene was released from hazardous waste sites in northeast Denver, and contaminated groundwater was identified through multiple plumes in this area. The water district in this area of northeast Denver detected contamination in source wells used by the water district was detected n 1981.

The water district in this area obtained approximately 85 percent of its groundwater from seven alluvial wells in this area. Contaminated water was pumped into the distribution system, and it was distributed in a relatively complex pattern.

Different TCE concentrations from different source wells contributed to different portions of the system.

Concentrations of trichloroethylene peaked in most of the district wells between 1985 and 1986, and treatment to remove organic chemicals from the water distribution system was initiated in February of 1986. So there was about at least a 5-year period where contaminated groundwater was pumped through the municipal water supply.

This map shows a diagram of this area in northeast Denver, Colorado. The outline here depicts the boundaries of the municipal water district. To the right of the slide is the Rocky Mountain Arsenal, which is a Superfund hazardous waste site, a location at which pesticides, chemical weapons, and a variety of other chemicals, dibromochloropropane, were manufactured over a number of years.

The industrial waste was dumped into unlined evaporation ponds, contaminants leached into the groundwater, and the groundwater was transported off site. This is another Superfund

hazardous waste site. There are about five hazardous waste sites in this area, in about a five-mile radius, three of which are Superfund sites.

The Chemical Sales Corporation site is another Superfund site in close proximity to the study area. TCE was used extensively as a degreasing agent at this site as well, and the use of that agent resulted in groundwater contamination. Right along the northwest boundary of the water district is the South Platte River, and it runs in a northeasterly direction. The groundwater flow in this area is to the north and slightly to the northwest.

This slide shows TCE contamination that was detected in some of the municipal source wells. over a period of several years. You can see that it peaked in different wells at different times, and it just gives you a sense of what the various concentrations were over time.

Okay; we have conducted, over the period of more than a decade, several investigations of

health conditions of the populations that are in direct vicinity of the Rocky Mountain Arsenal. In 1991, we measured neurobehavioral performance in 204 adults from this area who lived in close proximity to the Rocky Mountain Arsenal for at least two years.

This area is densely settled, so it would have been impractical and prohibitively costly to study every individual in this area. So for this cross-sectional study design, we randomly selected housing blocks along the densely settled boundaries to the northwest and west of the Rocky Mountain Arsenal. We conducted a door-to-door census of each individual in those randomly selected blocks.

From this, we generated an age and gender stratified sample frame of eligible individuals for the study, and from there, we randomly selected individuals for recruitment and participation in the study. Our participation rate in this phase of the investigation was 78 percent.

From this frame of individuals in which neurobehavioral testing was performed, in 1991,

there were 184 persons who resided within the water district who had undergone the neurobehavioral testing. So 184 individuals were served by the municipal water supply that we wanted to evaluate.

I mentioned earlier that the remediation in the municipal water supply began in 1985, so individuals who moved into the area between 1985 and 1991 were excluded from this analysis. The final sample, then, resulted in 143 individuals who had resided in the water district since 1985 at the time that they were tested in 1991.

To investigate neurotoxicity, we used the neurobehavioral core test battery. It is a battery of tests that was developed by consensus among neurologists and neurotoxicologists who worked in this area at a meeting that was held by the World Health Organization. It has since been extensively validated.

The battery consists of six neurobehavioral tests that measure different aspects of neurobehavioral performance, psychomotor function, memory, and combinations of those various

skills. They are considered very sensitive to the effects of neurotoxins, including solvents, such as trichloroethylene, solvents and solvent mixtures as well as other neurotoxins such as metals and pesticides.

So all of these tests were selected a priori for their potential relationship with the neurotoxins that were known to be present at these sites.

Six of these tests measure neurobehavioral performance. This is a measure of visual acuity or visual perception, and the other tests include the profile of mood states which is a marker associated with depression.

I want to also mention that we used a standardized protocol that had already been developed by the World Health Organization and ATSDR for delivery of these tests. Each of them, we had three interviewers. Each interviewer was extensively trained. They used the same protocol. We offered the tests in Spanish

to those who were more comfortable with Spanish as their native language.

I forgot to mention that the study population, the source population, has very low educational attainment. Approximately 80 percent of the population had an education less than 12 years; approximately 40 percent of the population had a family income less than $20,000 per year, and approximately 20 percent of the population was Hispanic.

This slide shows you the six different categories in the profile of mood states questionnaire. There are six outcomes that are generated as scores, tension, anxiety, depression, dejection, vigor, anger, hostility, fatigue and confusion. Each one of the tests that I showed you on the previous slide and this slide generate a numerical score, and so, each of those outcomes can be analyzed as a continuous variable.

Okay; to evaluate exposure in this study, we used a geographic information system. It was used in conjunction with a hydraulic simulation

model known as EPANET developed by the USEPA. We used these GIS and EPANET models to reconstruct hydraulic and water quality conditions within the water district.

1985 values of trichloroethylene in the source wells and the hydraulic parameters of the water distribution system in 1985 were used in our modeling. These values were chosen because they were representative of the entire time period during which we believe the bulk of the exposure occurred.

To do this, a digitized map of the pipe segments and junctions, valves and storage tanks was created in the GIS, and then, a simulation of the 48-hour hydraulic performance was used to calculate coefficients for each of 48 nodes within the water distribution system, and then, water demand was estimated at each of the nodes to define the geographic boundaries of the water demand within the system.

And then, finally, TCE concentrations that were present in the source wells at that time

were assigned to individuals in the water district based on residence within polygons that were then mapped to census blocks.

And again, this slide is just another depiction of the water distribution system; the location of the source wells are here, and the yellow represents a skeletonized map of the water distribution system, and here's the Rocky Mountain Arsenal boundaries.

And then, here are the results of the water distribution simulation and the assignment of exposure categories within the water distribution system. The white polygons represent TCE exposures less than 5 parts per billion. The yellow polygons represent TCE exposures between 5 and 10 ppb. The orange polygons represent exposures between 10 and 15 parts per billion, and the red polygon represents the high exposure category of greater than 15 micrograms per liter of TCE.

Okay; so, to perform the statistical analyses, we first screened 38 questionnaire items that were obtained at the same time that the

neurobehavioral testing was performed. We asked individuals questions concerning factors that may influence their neurobehavioral performance, including educational attainment, occupational history, hobbies. All the questions that we screened were basically selected a priori to be associated with neurobehavioral outcomes due to exposure to pesticides, solvents or heavy metals.

We screened each one of the items individually, then, to determine whether or not they were associated with TCE exposure. Potential confounding items if they were associated with the exposure categories univariately at a p less than 0.10 level of statistical significance.

Then, to test hypotheses concerning the relationship between potential TCE exposures and neurobehavioral outcomes, we used generalized linear models to calculate adjusted means, least squares means, for each neurobehavioral test across categories of TCE exposure, and we compared the high and the low exposure categories statistically.

Least squares means were used to adjust for the effects of education, smoking, alcohol and seafood consumption. These are four potentially confounding factors that we identified here in our screening stage of the analysis. And finally, we repeated this analysis with stratification by alcohol consumption to look for effect modification.

This slide represents some demographic information about the study population. There were about 20 percent of the population had estimated TCE exposures below 5 parts per billion; 23 and 43 percent of the population had intermediate exposures; and then, about 14 percent of the population had estimated exposures above 15 parts per billion. This results in estimates of about 80 percent of the entire study population having exposures through their water supply greater than the Safe Drinking Water Act maximum contamination limit of 5 parts per billion.

The distribution of age increased somewhat across the four Exposure categories. Educational

attainment decreased slightly across categories of exposure, and duration of residence increased slightly across exposure categories. And finally note that alcohol consumption slightly decreased as we went from low levels of estimated solvent exposure to high levels of exposure.

Okay; this next slide shows the results of the neurobehavioral test battery that were performed. Again, this first column of numbers represents the percent difference between the low and high exposure group, and the p value associated with the comparison of low and high exposure groups. These are--the comparisons again were adjusted for education, smoking, seafood and alcohol consumption.

And these results show about a 10 to 20 percent difference in scores, a decrement in these neurobehavioral scores across the various tests that were performed and a slight increase in reaction time. The change in direction across exposure categories is what one would expect to see if

there were neurotoxicity associated with expsoure. An increase in reaction time is also the direction that you would expect to see if there was neurotoxicity, although you can see that none of these tests attain statistical significance, although three of the tests, contrast sensitivity C, contrast sensitivity D and the digit symbol test, were of marginal statistical significance.

This slide shows the results for the profile of mood state scores for TCE exposures, and it's organized in the same manner as the previous slide. As you can see, there were no statistically significant increased scores for changes in mood, although depression with an 83 percent difference between the high and low exposure groups was suggested again.

The next slide shows the analysis as it was performed with stratification by alcohol consumption. So to do this analysis, we divided the entire population into groups of individuals who consumed alcohol and those who did not consume alcohol. There were 55 individuals who reported no

alcohol consumption whatsoever and another 84 individuals who did report some alcohol consumption; four individuals did not respond to that question.

As you can see, there were absolutely no TCE-related effects in individuals who did not consume alcohol, whereas, among individuals who reported alcohol consumption, there were statistically significant differences between the low and high TCE exposure groups, and those occurred for the Benton visual retention test, the digit symbol, digit span, digit span forward, and simple reaction time. and the digit span backward also showed a 24 percent difference between high and low exposure, although it was not statistically significant.

This slide shows the results, now, of --oh, I should mention in the previous that we saw in the overall analysis with contrast sensitivity C and D, we saw a suggestion of an effect associated with exposure. We did not see any statistically significant change in contrast sensitivity C or D

when we stratified by alcohol consumption, so those results are not presented here.

This slide shows the profile of mood state scores stratified by alcohol consumption; again, among individuals who did not report any alcohol consumption, we saw no increase in affect and no statistically significant differences associated with solvent exposures, whereas, in the alcohol consumption group, we saw highly statistically significant changes in confusion and depression scores as well as increased tension and increased anger scores among those who consumed alcohol.

So the uncertainties associated with our results: first of all, the exposure, there are uncertainties that surround the exposure estimation certainly. We did attempt to validate our simulation of distribution of solvents, of TCE, through the municipal water supply. We used 1984 data on TCE values that were present in the source wells and the same simulation to look at exposures as they might have occurred in 1984.

And what we saw is we saw changes in TCE

concentrations that were estimated using the 1984 data, but the relative ranking of the various polygons in the high and medium and low exposure categories did not change substantively during that period. So this gave us some confidence that our simulation model was indeed predicting exposures throughout the duration of exposure that we were interested in.

Another uncertainty that is certainly that there are other contaminates present in these source wells. TCE was not the only contaminant, although it was present at some of the highest concentrations in most of the wells that were tested. Other chemicals included tetrachloroethylene, dichloroethylene, trichloroethane among others.

Our cumulative exposures were not estimated in this study, and so, it is not known what the effect might have been if we had been able to evaluate that. Latency assumptions are uncertain in terms of how long someone would have to consume water at these levels to be able to

detect neurological changes.

The use of bottled water, showering and bathing and other sources of exposure to TCE were not evaluated. We simply looked at exposure through tap water sources through presumably direct consumption. The low SES in this region sort of argues against bottled water as being a significant confounding factor.

There is always the chance for residual confounding in any epidemiologics investigation, and this is no exception. The reporting of street drugs is a sensitive question, and for this to have been an effect, the distribution of the use of illicit drugs would have had to have been distributed in the same manner as the pattern of exposure occurred throughout the municipal distribution system, so we feel that this is probably unlikely to have occurred.

Information bias is also possible for questions such as alcohol consumption. People may be more sensitive to that and not willing to report that accurately, and that is a possibility,

although we did consistently observe using several different alcohol related questions that there were 55 individuals who did not report any alcohol consumption. So those are certainly things to take under consideration.

So in conclusion, the findings add to evidence that low-dose exposure to TCE can reduce neurobehavioral performance. Our results are compatible with other studies. The estimated exposures in this study are lower than what has typically been reported for other investigations. The mood state abnormalities are considered an early sign of neurotoxicity. The interaction with alcohol is important for residential as well as occupational exposures. The mechanism whereby this might happen is fairly complex. It could involve altered metabolism or increased concentrations or increased blood concentrations of trichloroethylene through altering metabolic processes.

There is some evidence that TCE may act competitively with, given in an acute bolus dose, whereas decreased clearance rates may also

be--there's evidence for decreased clearance as another mechanism for increasing TCE exposures.

Finally, I think our results demonstrate the utility of using GIS-based modeling, spatial distribution of contamination through a municipal water system. It's one of the few examples of this ever to my knowledge. We used in a previous analysis where we looked at potential exposures based on proximity to the waste site, we found no neurobehavioral decrements in the populations living in the vicinity of these waste sites, and that is probably due to nondifferential misclassification, which can bias effects estimates toward the null.

So, with that, I conclude my presentation and thank you for your attention.

[Applause.]

DR. PREUSS: Questions?

QUESTION: I need to understand your design a little better. Your making your measurements some years after the exposure is over with. Is that correct?

DR. BURCH: No, no, the exposures occurred--the TCE was detected in the source wells in 1981, starting in 1981. In 1985, they initiated a remediation filtration system to take the chlorinated solvents out of their water. So the exposures occurred at least between 1981 and 1985. The testing occurred in 1991.

QUESTION: So some years after the exposure.

DR. BURCH: Yes.

QUESTION: So these are residual effects, and I'm trying to grasp your argument about effects on metabolism and so on and so forth, on something--I don't think anybody's showing a half life of TCE or any of its metabolites that would be six or seven years long. And I don't know --and another thing I bring up is I don't know any precedent in the animal literature that would suggest you have residual effects even from high exposures to trichloroethylene in neurobehavioral. So I am a little confused about what all this means, I guess.

DR. BURCH: There are a couple of references in our paper about residual effects of solvents that are present up to a decade after exposure. And so you can--

QUESTION: At 15 parts per billion.

DR. BURCH: Not at 15 parts per billion.

QUESTION: Excuse me Jonathan Borak, Yale. I think it is very praiseworthy in the effort to enhance the distributional models. Most of the historical, ecological studies on TCE have actually broken down once the true distribution of the water has been better studied. And so, I think your effort is very praiseworthy.

I raise a different concern, however, which is that you have a huge number of comparisons that you've made, and you have liberally used the concept of statistical significance, and I think one should be cautious. There really needs to be an adjustment were made, those comparisons, this does not speak to a pattern of effects, but those conclusions which you describe as statistically significant, most would in fact not be. And I

think that the pattern is interesting, but I think the conclusions are overly aggressive.

DR. BURCH: Well one can argue about the need for adjustment based on the number of statistical tests that were performed. Certainly, we selected all of the neurobehavioral tests a priori, and if you wanted to back off from the statistical significance, I think you can just look at the effect estimates, the percent differences, and see that there's clearly a trend toward a decrement in neurotoxicity.

We did not see really significant effects in the main analysis. It was only really when we looked at the stratified analysis where we look for effects among those who consumed alcohol, and so, I think it's clear evidence for effect modification in this case.

QUESTION: I mean to suggest that I think you raise interesting hypotheses, and I think that the problem is that having raised them in this kind of a context, it begs replication before one can actually draw inferences of any certainty, and

particularly, the interaction with the ethanol raises another new set of considerations. And I think that this is very informative and it's illuminating, but I think it's not conclusive.

And I would just raise the caution, given the structure and design of the study and the large number of comparisons that the use of the concept of statistical significance in this context is perhaps too aggressive.

DR. BURCH: Well, I certainly agree that there is a need to study this further, and I appreciate your comment.

DR. PREUSS: Dr. Lipscomb?

QUESTION: I was wondering if you might provide some context. A lot of the information you collected and analyzed and you interpreted as being marginally significant. I believe there were three effects noted on one slide. Could you maybe inform us as to the magnitude of the difference?

DR. BURCH: I'm not sure what you're getting at.

QUESTION: What exactly did you measure?

Your slide used acronyms that I don't understand, and I don't know the types of measurements that you were making. The contrast in the digit; thank you.

DR. BURCH: You have to distinguish--there's a pattern of numbers embedded in another pattern. You have to distinguish the number that's embedded in the pattern. Perhaps you have seen this. This is what the tests measure. The digit symbol is you're given a series of symbols, numbers, and basically, the symbols are presented, and then, you have to remember what symbol corresponds to what number, and you have to generate the numbers. The digit span is a test where you are given a series of numbers to memorize in a certain amount of time to measure them. You start with two or three digits, and it goes up to increasing numbers of digits. You have a certain amount of time to memorize them, and then, you have to repeat what the order is in which the numbers appeared. And you just do these tests until you fail. The Santa Ana test is a test of psychomotor dexterity where you have to take a peg out of a

hole and you turn it around, and you have to put it back in the hole fast as you can within a certain period of time. Reaction time is simply a light is presented, and you have to push a button as soon as you see the light. So it is a measure of neural processing. The Benton is a visual retention test. And because of the population we were dealing with, we didn't use a computer for most of this. We'd give people a paper and pencil and place a dot in the center of a circle on a piece of paper. All of these were done because of the educational attainment of the population. We used pen and paper and using other types of devices. Does that help?

QUESTION: It does.

QUESTION: Bill Scott, Dow Chem. Two questions, really. Did you consider evaluation of other nontraditional factors in this study such as drug use and gang activity? With drug use, gang activity, some of the boundaries are fairly defined; so are gang boundaries. I'm looking at Dr. Benson here; the potential exposure from vapor

intrusion into basements, things like that. I'm not exactly--

DR. BURCH: Well to answer the first question, we did not look at gang activity or drug use. The questions were screened in the same manner that all of the other confounding variables could fall into a residual confounding category. The prevalence of gang members, I'm not sure about. As far as exposure through the inhalation pathway through the groundwater in this area, that's a difficult one to ascertain. I mean, it would have been volatilization; it could have been through the tap water, so showering would have been a major source of exposure. There was a change in the water distribution system in 1985. There were also a series of groundwater extraction wells within the boundary over a number of years ago. So the groundwater source is also being captured, the source of groundwater contamination was being captured as well. So the window of opportunity to study individuals their TCE levels and the airborne TCE levels in their basements, I think, may have

already passed.

DR. PREUSS: Another question?

QUESTION: Yes.

DR. PREUSS: Please.

QUESTION: Michael, along the lines of Dr. Borak's questions, with a lot of these tests, if we have better information on specificity, would it not be better to use these rather than using epidemiological studies that you would rely on certain tests more than others rather than having ones that we can trust more than others?

DR. BURCH: They were all selected based on their validation in previous studies. It requires a number of different psychological and neurological facilities to be intact.

QUESTION: The education level of these people was generally low. Is it possible that this has affected the results?

DR. BURCH: Certainly, education is an important factor to consider, absolutely.

QUESTION: This is Dick Bull again with MoBull. I noticed I think in the previous slide

that the respondents in the last group on your graph, were they not about five years older? Or did I just make that up?

DR. BURCH: Excuse me?

QUESTION: The age distribution of the people in the highest group was 55, and I'm looking at the tests that came up closest to being significant as involving visual acuity, and it was a long time ago when I was 55, but I do remember that there were some things that were going on with my eyes. Have any of these been normalized with age?

DR. BURCH: We performed a completely separate analysis where we adjusted for a priori confounding factors, and we obtained essentially the same results.

DR. PREUSS: Dr. Wang?

DR. WANG: Simply, just by looking at this figure, we understand that the number of people who are above 15 parts per billion and less than 5 parts per billion were relatively small. So I don't know whether when you adjust so many

potential confounders, is it--I have two questions. One is that is still stable; the other is why not just try to test for trend and compare?

DR. BURCH: That is a very good suggestion. In fact, we did test for trend. The tests for trend essentially come out the same as the high low comparisons; they're basically the same as what you see there.

DR. PREUSS: Well, let me thank you very much. We had extra time at the end; are there any of you who didn't have a chance to ask on any of the other papers? We have the opportunity now. If not, all of you are certainly welcome to speak with one another. So let's see if there are some. I see that there's at least one. So could you again identify yourself?

QUESTION: This is for Dr. Hansen. My name is Perry Cohen from the New Jersey Department of Health. I'm wondering about people who have been employed under about three months or so, and the question is do you have in the Scandinavian countries like we've noticed in the U.S. that

oftentimes, people are asked to do very dirty tasks, and they are short-term employees, and they receive the highest exposures, so that in fact, dose-response may be a little bit confused?

DR. HANSEN: Yes; I think it's the same overall in the Scandinavian countries may have the pictures of the people who are newly employed in a company may have the hardest work tasks, I think, but I don't think we can set the limit to only three months or six months or one year. But there is a tendency that if you are long-term employees, you get a better type of work when you have been there for a long period.

DR. PREUSS: Can't move around here without being miked up. So are there any other questions?

[No response.]

DR. PREUSS: If not, we are ahead of schedule. The schedule calls for us to start again at 1:30. I suggest we take an hour and 15 minutes for lunch. And so, we will see you all back here at 1:00, then.

[Whereupon, at 11:46 a.m., the meeting recessed for lunch, to reconvene at 1:00 p.m., this same day.]

A F T E R N O O N S E S S I O N

[1:07 p.m.]

DR. BUSSARD: My name is David Bussard. I'm the director of the Washington division of NCEA. Peter and I are going to do tag team a little bit this afternoon. He has another commitment to juggle this afternoon.

Thank you for coming back after lunch. It's always a struggle whether you reward the people who came back on time or whether you give everybody some slack. So we'll start just a couple minutes late.

We'd like to introduce Dr. Shiao from the National Cancer Institute. He's going to talk about VHL alterations in renal tumorigenesis, and it's all yours. Thank you.

DR. SHIAO: Thank you.

Thanks for the organizers inviting me to give a talk. Personally, I haven't done any project on the trichloroethylene, but since this is some evidence of link of trichloroethylene linked to kidney tumor and also other types of tumors, and also VHL

has been examined in kidney tumors and associated with trichloroethylene exposure, so I will try to bring those data together along with my personal findings from other VHL studies. And, and I'll try to give you an overview of the VHL in the renal tumorigenesis.

The VHL, the name came from the von Hippel-Lindau disease. It's a hereditary human Disease linked to VHL mutations, and the patient tend to develop various kinds of a tumors, including renal cell carcinomas. And ,VHL mutations also occur very frequently in the sporadic renal cell carcinomas, especially the clear cell type. The type of mutations include, loss of heterozygosity, it's a type of technique, you use a marker to differentiate the gene loss at a specific loci.

And since VHL is located at this chromosome 3P25 region, loss of heterozygosity would be an indication of the VHL gene loss, and it occurs in more than 90 percent of renal cell carcinomas, especially the clear cell phenotype. And, mutations

in the VHL coding region occur between 30 to 60 percent depending on which studies; some studies show low frequency and some show high frequency.

And hypermethylation also occurs in the VHL promoter region, and this is in up to 19 percent of the cases. And for this familial VHL disease can be classified according to different tumor combinations; for type I, in yellow, you can see the family members can develop either renal cell carcinoma, central nervous hemangioblastoma, retinal hemangioblastoma and pancreatic tumor.

And for type II, that's the magenta color you can see that there's a couple of other combinations; I want to point out that the type I and type II-B include the renal cell carcinoma. See both the yellow and the blue color. They tend to have the deletion and frameshift mutations in the families. And for the missense mutation, it tends to occur in the family with pheochromocytoma, and so, there is some genotypic and phenotypic correlation.

The family tends to have a deletion or frameshift alteration. They tend to have mo re renal cell carcinoma. So this is an indication that this is a different type of VHL alteration. Frameshift, that is a type of alteration that is the result of a truncated protein. So either deletion or frameshift, they correlate with an increase in risk of renal cell carcinoma but not the missense type.

And for sporadic renal cell carcinoma primarily occurs in the clear cell, as I pointed out in the beginning; more than 50 percent of mutations are the deletion or frameshift type of VHL alteration. The percentage for missense mutation is relatively low frequency, but it depends on the studies; some report low frequency, some report high frequency and some ranging from 30 percent to 70 percent.

And the other type of renal cell carcinoma is the papillary, chromophobic and oncocytic tumors. They are not very frequent overall; in all kidney tumors, they don't have a frequent VHL mutation. This is some percentage, but very low frequency, in the

papillary or chromophobic tumors, and they can range from 0 percent to 5, or 6 percent of the VHL alteration.

And with that in mind, VHL mutation is so Frequent in the common type of renal tumors. We may make the assumption see that VHL may play a role in the tumorigenesis, either in the initiation or in the progression. If that is the case, then we would associate that VHL alteration will associate with the tumor pathology, but on the contrary, there is no consistent data to show association of VHL gene alteration with associated with the tumor stage, nuclear grade or metastasis.

And to our surprise, the mutation, the gene, including the deletion, frameshift and missense mutations, tend to associate with a better cancer-free or cancer-specific survival. We are talking about the renal cancer-free or renal cancer-specific survival. So that is contradictory to our assumption that VHL mutation plays a role in the tumorigenesis, how come it associates with a better survival?

And there's a similar phenomenon that also occurs in the other type of gene alteration called

microsatellite instability. It's a type of DNA sequences, either dinucleotide, such as CACACA or GTGTGT repeats, trinucotide or tetranucotide; we would classify that as a microsatellite sequence present in the human genome. Very frequently, they tend to show a changing of the number of the repeat, and the micosatellite is also detected in very early tumor stage in many different kinds of tumors, including colon cancer and renal cancer, but the micocetalide instability is also associated with the better survival.

So it may be that VHL mutation is an indication that maybe VHL plays a role in--maybe VHL is an indication of tumor development but does not necessarily play a role in the tumor initiation process. And in addition to the gene alteration, we also look for the protein expression change. And there is large evidence of the VHL mutation only occurring in the clear cell phenotype, protein change had not been examined.

In our earlier study, we also examined the VHL protein in the non-clear cell type tumor in the rat kidney. And we observed the down-regulation of

the VHL protein in the rat non-clear cell kidney tumors, so we expanded that study to the human population.

Consistently, we observed the down-regulation of the VHL in the tumor area. This is the tumor area, and this is the nonneoplastic area, showing intense brown signal. In nonneoplastic area adjacent to the tumor, you can see the tubules were stained positively but the glomeruli were negative. And some of the tumors staining positively, we observed a different different pattern of expression.

Some tumors express the protein on the membrane area. Some express in the cytosolic area. And this is an indication of a different types of VHL alterations may have a distinct functions, and then, some of the altered proteins may shift, some of the altered proteins may migrate to the membrane area; some may stay in the cytoplasm area.

And when we correlate the VHL protein expression profile to the tumor pathology and also

the genotype, we observe the significant association of the membrane staining to the missense type of alteration. And here, the nuclear grade is not associated with the membrane staining, but you can see there is a trend toward higher grade tend to have a low percentage of membrane staining and a high percentage of either cytoplasm staining or no staining at all.

And the membrane staining is also associated with the tumor stage; especially membrane staining occurs at the early tumor stage. And in other studies for the VHL protein, other groups using a different antibody, they use a polyclonal antibody, they identify that VHL present in the nuclei and/or in the cytoplasm. And they categorize staining into two different groups: either the nuclear and/or cytoplasmic staining or no staining, and the positive staining is associated with the lower nuclear grade and the lower lower tumor stage consistent with our finding.

And also, they also show the nuclide cytoplasm staining--the patient has a better nuclei

cytoplasm staining, a better survival. So, from the data up to this point, we can tell that the VHL gene is not consistent--gene alteration is not consistently associated with the tumor pathology, but the protein is consistently associated with tumor pathology, indicating the protein expression may be a better marker than the gene alteration to predict not only the tumor pathology and also the patient's survival.

There is still some question about the initiation, and we know VHL gene alteration is very frequent, and the protein down-regulation or protein expression difference also very frequently occur in the kidney tumors, but that does not mean VHL alteration contributes to the tumor development.

From the earliest data, we know it is associated with the tumor stage, so that is the main indication that some VHL alterations are associated with progression,

but it is still not clear whether a VHL mutation eventually leads to tumor or not, especially from the VHL disease is a familial disease; even those families they have a VHL mutation, but not all of

them develop the kidney tumor, and also, not 100 percent of those patients develop any kind of tumor at all. It's 90 percent of penetrance when the VHL patients reach the age of 65, so this means there is some interaction of the gene and the environment, and the environment promotes the tumor development.

And with the presence of the VHL alteration, the environment played a very important role to lead to the tumor development. And also, there is some diversity of the VHL alteration, and they may have a different tumorigenic function.

And to answer whether VHL plays a role in the initiation or not, this is some evidence from the in vitro and in vivo studies. And this is a very complicated slide. But I'd like you to focus on the red color areas. The VHL has been shown involving the cell cycle regulation, down-regulated the cyclin D-1; this will inhibit the retinoblastoma gene and lead to the increase of gene expression and cell proliferation, and VHL cannot up-regulate P-27; it's a cyclin-dependent kinase inhibitor.

This yp-regulation inhibits cyclin-dependent kinase 2 (CDK2) and cycline E. And also, VHL can inhibit the TGF-alpha through the signalling pathway can either lead to the increase of HIF-1 and HIF-2 alpha; this hypoxia-inducible factors had been a linked to many tumor phenotypes, including the angiogenesis and tumor acidity.

Those cell proliferation or angiogenesis or acidity, they all play a very important role in the tumor progression or tumor development. And we reported a couple years ago, we introduced mutated VHL into the cell, into the rat nontransformed line. We also see the mitochondrial abnormality. I will come back to this later.

And the other evidence of the tumor development is involving the tumor progression is angiogenesis. VHL regulates the degradation of the hypoxia-inducible for factor alpha. And increase of hypoxia-inducible for factor alpha will lead to expression of vascular endothelial growth factor or erythropoietin. Those factors, they are all involved in the angiogenesis. And then, also, this

is some HIF-independent paths will lead to an increase of VEGF, vascular endothelial growth factor and lead to an angiogenic phenotype.

So VHL is involved in the angiogenesis. You can get some idea from this pathway how it is involved in angiogenesis. And from the animal study, peoples tried to demonstrate that VHL is involved in the tumor development. And so this is not --A couple of VHL-knockout animals have been developed. The homozygous VHL-knockout mice die at the gestation stage of 10.5 to 12.5 days, so it is embryonically lethal. And heterozygous mice are susceptible to develop vascular lesion, especially in the liver but not in the kidney in two or three knockout animal studies.

And this vascular lesion is basically just the proliferation is consistent with the angiogenic phenotype or observed in the familial VHL disease, but none of them are linked to this either renal cell carcinoma or pheochromocytoma or hemangioblastoma. Those tumors arise from the parenchymal cells.

So because of the homozygous knockout animal is embryonically lethal, so some groups have developed a conditional knockout. The conditional knockout mice carry VHL allele with the lox-p--the gene is flanked by the lox-p sequences. This lox recombination can be initiated by the creorecombinates through this recombination process, the result of a deletion of this allele, so the VHL will be deleted in a specific or selected organ rather than deleted in the very early stages of embryonic development.

So, there is no embryonic lethality problem here, so you can demonstrate whether the VHL loss in the target organ will lead to the tumor development or not. Consistent with the heterozygous knockout mice, an increase of vascular lesion is also observed, primarily in the liver and a small percentage of vascular lesions also occur in the heart, kidney and pancreas. But I have to emphasize this vascular lesion is just the proliferation; there's not any type of tumor associated with a family of VHL disease.

So, still come back to VHL alteration is

play a role in the tumor initiation or just the progression? And from the data so far, it looks like VHL plays a very important role in the progression. And this is a finding we probably should have covered years ago to link VHL framshift mutation to tumor initiation.

We introduced the altered VHL into the nontransformed rat kidney cell and tried to see with the presence of the altered VHL what kind of a phenotype occurs in this cell so that will give us some indication what the early change during the tumor development.

And when we transfect a cell with VHL, you can see that the VHL was localized to the mitochondria by immunogold electron microscopy; you can see the dots indicating the goal particles present in the mitochondria only, not in the nuclei or the other regions of the cytoplasm, and this is the other view of the cell transfected with the wild-type VHL. But when we transfect with the mutant VHL, you can see there's a large change of the mitochondria phenotype, and the arrow points to the crostaes. Am indication of the mitochondria, and you also can see some small mitochondria present adjacent to

this area.

And those small mitochondria, also, this type of a phenotype is also observed in the human renal cell carcinoma, especially the clear cell renal cell carcinoma; it tends to have a low number of the mitochondria and also small mitochondria. This is just the control without transfection, an indication of a low expression of endogenous VHL.

So this is some indication that VHL may play a role in the initiation, but I need to point out that from the familial VHL disease, we know only the deletion and frameshift are associated with renal cell carcinoma development. And from this study, we introduced the mutant VHL; also, the frameshift type of alteration, and we also try one transfection with just the missense mutation. We didn't see small mitochondria phenotype.

So there is some indication that VHL alterations can be classified into different categories, either frameshift, deletion, missense mutation, and some missense mutations may have a different tumorigenic potential and the same thing

for deletion and frameshift. They also have a different potential.

And besides, the VHL has been used to determine the role of the renal cell carcinoma development, and the VHL mutation spectrum can also be used as an indication of exposure. There's a summary of the different type of mutation spectra, which I compiled several years ago. The GC to AT, either G to A or C to T type of mutations are the most common type of mutations in human neoplasms can result from the deamination or alkylation, and some oxidated damage can also cause this type of mutation spectrum. And you can also see a different type of mutation spectrum linked to a different type of exposure, oxidative exposure or depurinization and some repair associated with the mutation.

And there is one major study on the VHL mutation spectrum linked to the TCE exposure. And this group, they linked high exposure of TCE to the hot spot mutation at the nucleotide 454 site and also link to higher percentage of a mutation, and also, the number of mutations increased in the high exposure group. And for the GC to AT mutation, the

percentage is also very high compared to the overall. This is from mutation database, VHL mutation database published in 2000, a relative GC to AT mutation higher in the TCE exposure gr oup and also percentage of missense also increased.

And so, the conclusion is there is still no direct evidence VHL alteration initiated the renal tumorigenesis, but there is evidence involving the tumor progression.

And the different type of alteration has a different tumorigenic potential, and the comparison of a mutation spectrum has the potential to identify a specific base change, but more study needs to be done to have sufficient statistical power. And also, of course, coexposure to things such as smoking, hypertension, obesity, chronic renal disease, they are all known risk factors for renal cells. They need to also be examined along with the TCE.

Thanks for your attention.

[Applause.]

DR. BUSSARD: Thank you. We have time if

there are a few questions.

[No response.]

DR. BUSSARD: Okay; looks like there aren't any. Thank you very much.

We would like to move on, then, and invite Dr. Larry Lash to talk about metabolism and mechanisms of renal cellular injury.

DR. LASH: Okay; well, I want to thank the EPA for inviting me, organizing the meeting. And I was sort of given a very broad charge. I was asked to sort of provide information on nine of our papers from the last three years covering the areas of metabolism and the mechanism of action of DCVC. So what I'm going to try to touch on since it's hard to really cover all this in much detail in 35 minutes is just to give some examples, and certainly, if there is time for questions afterwards.

But I'll discuss, as far as metabolism, a little bit about differences in the roles of P450 versus GST, glutathione S-transferase, the species differences and tissue differences, in terms of

kidney metabolism, a little bit about differences in the role of beta-lyase-dependent activation versus FMO, which is S-oxidase activity, and also sort of the outlier in terms of organs and topics but relevant to metabolism is some recent studies that were published in 2002 and 2003 that were a collaboration with Dr. Poh-Gek Forkert at Queens University looking at metabolism and toxicity in the male reproductive system in the mouse, and we have some human data and some non-human primate data as well.

And then, the bulk of the talk will deal with more recent data, and I guess it's all sort of new since publication of the Environmental Health Perspectives supplement in 2000 looking at DCVC, the cysteine conjugate metabolite, how it induces renal toxicity, a little data in rat, mouse and human, and then most of it, actually, I'll focus on more recent studies in primary cultures of human proximal tubular cells and assessing differences in the role of FMO and beta-lyase in bioactivation and then finally a

little bit about a study of in vivo evidence for function of the GST pathway.

This slide just gives an overall schematic of the metabolism. And of course, here's TCE or tri, as I have often abbreviated it, and essentially, there are two general pathways, the P-450 or the glutathione S-transferase pathway, and some of the key metabolites that are generated by P-450 include initially chloral hydrate, which has been associated with effects in the lung in the mouse; also, trichloracetic acid and dichloroacetic acid, which you will hear more about from Dick Bull and Mike Pereira later this afternoon.

The kidney pathway, or I should say the GST pathway, which can be initiated in either the liver or the kidney is what is presumed and evidence indicates is associated with all of the toxic effects associated with the kidney. And this includes glutathione conjugation to form the glutathione conjugate. It's processed to the cysteine conjugate, which functions as a branch

point where you can either have formation of the mercapturate down here, although this is reversible by deacetylation and either the beta-lyase or FMO to generate the reactive species.

So the first study I want to talk a bit about is one that we published in 2001 where we were looking at trying to define any potential role for P450 in the kidney in the rat. And what this shows is the P450 reaction, the epoxide, dichloroacylchloride or chloral, and in the liver, the primary players in the rat that are believed to be involved are CYP2E1, 2B12, 2C11 and to a lesser extent 1A1/2, and there was really nothing known about the kidney.

We do know from studies that we published in the mid-nineties that many of the P450-derived metabolites, when kidney cells in vitro are exposed to these, there's essentially no effect. But an important point regarding metabolism is how P450 metabolism might influence glutathione-dependent metabolism, since that is a species believed to be important in generating the nephrotoxicant.

So, CYP2E1 has always been considered the main player, and in rats, rat kidney, it is readily detectable, and I will show a little data on that. It's the major P450 in proximal tubular cells for TCE. The kidney also has a high amount of P450s in the 4A family, 4A11 in humans, 4A2 in rats that are involved in arachidonic acid metabolism, for example, but the levels in rat kidneys are much lower than in the liver.

In humans, it's kind of unclear what the implications are, but there's no detectable P450 2E1 by either an activity assay or Western Blot, and there's virtually no--we were not able to detect, except in one sample out of about a dozen, any P-450-dependent metabolism of trichloroethylene. So likely, it's not going to be important in humans but may--and, in fact, we have some unpublished data that it may influence disposition in the rat.

This is just a couple of slides to illustrate some of the tissue and species differences. For instance, pyridine is a well-known inducer

of CYP2E1. And this shows a Western Blot for rat liver, and you see a nice about fivefold induction in amount of protein, and in kidney, it's, of course, much less; it's present, but you also get about a twofold induction in kidney microsomes.

Here, looking at clofibrate as an inducer, which interestingly does not induce the liver 2E1 but does induce about two and a half fold in the kidney, and 2C11, which is probably, in the rat kidney, the secondary P450 enzyme involved; again, clofibrate had no effect in the liver but produced about a twofold induction in protein content in the kidney microsomes.

Now, to sort of shift gears a bit to talk about the metabolism in the male reproductive tract and sort of--although it's still consistent, sort of, with the general focus on extrahepatic metabolism; this is a study that was a collaboration with Dr. Poh-Gek Forkert at Queens University, and this is looking at CYP2E1 expression by Western Blot analysis. The first three lanes are in mouse

testes; 10, 25, 50-, micrograms of protein; then, in epididymis, where it's found at apparently a much higher concentration, 5, 10 and 25; and then, for comparison, when 2 micrograms of liver protein was loaded, we actually had the largest response.

So it's present there; and this is an immunofluorescent staining in the epididymis. You can see positive staining in the epithelial cells and in the testes in the leydig cells. So the protein is there, and in--this is again in mouse. When we look at quantifying metabolism, the P-450-derived metabolite chloral is formed in a time and NADPH dependent manner in the testes and in the epididymis, and consistent with the protein expression, the amount of metabolite formed in the epididymis is about two and a half fold, threefold higher than that in the testes, and this corresponds with para-nitrophenol hydroxylase activity.

So it has implications, then, for potential questions of infertility in males exposed to trichloroethylene, and this is

again from the mouse study, just illustrating mice were exposed; and again, these are very high levels; 1,000 parts per million TCE by inhalation, six hours per day, five days a week for four weeks, and you can see here's control and then significant morphological damage to the epididymis.

Now, in another study, and th is was published in 2003 in Drug Metabolism and Disposition, Dr. Forkert, again, at Queens University in Ontario, obtained access to seminal fluid samples from workers exposed to very high levels. And the point, I think, that's very interesting here is the huge variation. There are eight subjects, and this is showing the parent compound, and you can see it varies in seminal fluid; it varies from a low of 20 in one individual all the way up to over 5,000 picograms per sample.

And then, as far as the metabolites, again, there is a huge degree of variation. And I believe that the time from exposure was pretty much the same for all the individuals, so it's not a difference of the time of exposure. And the type

of work that each did was the same as well, so it's thought that the exposure levels were similar, but again, if you look at chloral hydrate levels, they vary over almost 150 fold from a low of about 60 to 70; one individual at 1,700 picograms per extract. Similarly, trichloroethanol levels varied over tenfold, and TCA was only detectable in one individual where it was very high, but it was below the limit of detection in the other seven. And similarly, DCA was detected in two individuals at very high levels and not detected in six others.

So, obviously, an important issue, particularly if you're looking at development of a biomarker, for example, for exposure is it's going to have different implications for different individuals depending on their genetics. And we don't have knowledge yet about what determines the levels. And this is just a small study that was conducted.

Now, 2E1 is also present in the human testes and epididymis. And shown here in the testes, the arrows indicate that it's found in the

Leydig cells, and in the epididymis, it's found in the epithelium, so similar to the mouse. And this is immunofluorescent staining from monkey epididymis, showing 2E1 presence, so a non-human primate, so there's consistency among the species.

So some of the conclusions from this work is that CYP2E1, which is the major P-450 enzyme that metabolizes TCE, is present in testes of mouse, a non-human primate and humans. The activity and expression are highest in the epididymis; histopathology is observed in epididymis of mice exposed to albeit a very high level of TCE, but humans exposed occupationally to relatively high levels exhibit both TCE and some of its metabolites in seminal fluid, although there is again a great deal of interindividual variation.

And so, the data are consistent with a role for 2E1 in both experimental animals, non-human primates and humans in bioactivation of TCE leading to testicular toxicity, but again, everything must be put into the proper context of dose. And so, again, I think additional studies

are needed to establish that.

So, next, sort of to move on back to the kidney and to the GST pathway, this was a table I published just summarizing rounded-off general rates of metabolism comparing rat and human for overall dealing with a high level of TCE, about 1 millimolar and comparing rates of metabolism, and you can see for rats versus humans that there's not a large difference between P450 or GST. Gamma-glutamyl transferase is much higher in the rat.

The big difference would appear to be in beta-lyase. So it's possible that one of the reasons why male rats exhibit a higher susceptibility to kidney toxicity is due to the higher levels relative to humans of this enzyme, but I think there's a lot more to it than that.

One of the things we looked at recently was expression levels of different GST isoforms in rat kidney and proximal tubular cells in humans. In the rat, the only isoform expressed is GST alpha, and this Western Blot shows--this is a positive control of purified alpha 2-2, then cortical cells, proximal

tubular cells and distal cells. Mu and pi are not expressed in the rat kidney.

In the human kidney, interesting ly, there's a much more diverse expression. Both GST -alpha, pi and theta are expressed. And what's interesting, though, and, of course, this is a limited sample size; we obtain fresh kidneys. These are kidneys that are not--determined not to be usable for transplant, and we obtain them usually within 24 hours of coming from the donor, and we've used them as freshly -isolated cells to study metabolism or do expression stud ies like this, and we've put them into primary culture and studied more mechanisms of injury and metabolism.

So in these limited number of samples, what we see is for GST alpha, there was a modest degree of interindividual variation. Each of these is a different donor, different sample. For GST pi in these 7 or 8 samples, the interesting thing is that one individual had no detectable GST pi; one had extremely low; and then, comparing the low one to the high one, there's over 100 -fold variation.

So there's potentially a polymorphism there. And GST theta expression levels were pretty consistent in the samples, and we've done some additional samples as well that exhibit similar types of data.

So what the implications are for TCE, however, is not clear, since we don't really know any differences between the ability of each of these isoforms to metabolize TCE or what implications that may have. But I think it very potentially can, since we know, for example, that GST pi or rather the GST mu null variant that is present in about 40 percent of individuals leads to a markedly different susceptibility to colon carcinogenesis, for example, and bladder cancer from different carcinogens, so it's possible that this may influence TCE metabolism and bioactivation.

So then, in this slide, I wanted to summarize a little data from a paper we published in 2001 comparing acute toxicity in male and fema le Fischer rats. And these were comparing in rat kidney cells and hepatocytes. We use release of a cytosolic enzyme lactate dehydrogenase as a measure of acute

necrosis.

And these are fairly high levels, so they're obviously not going to be relevant for the human exposures, but our later studies that we've done in culture are more relevant levels, but it gives you some idea of potential differences. We exposed to either the parent compound, the glutathione conjugate or the cysteine conjugate, and fairly consistently, the cells from the male rats exhibited a higher degree of acute toxicity than those from the female rats.

And the kidney cells--now in the hepatocytes, I sort of show this to make the point that actually, DCVC, the sort of penultimate nephrotoxic metabolite, is just as cytotoxic to hepatocytes as to kidney cells. However, in vivo, you never see any liver injury. So that's simply an in vitro type of response. But again, the males seem to exhibit more injury. So there are gender-dependent differences, even in the rats.

So most of the rest of the time, I want to talk about more recent work on looking at what

might be called environmentally relevant exposures of primary cultures of human kidney cells to DCVC and to trichloroethylene, and this just lists the medium that we use for culture, and it's a fairly standard procedure, DME-M/F12 medium. And then, you add a variety of supplements that we use, so-called serum-free, hormonally-defined conditions, and this provides optimization of differentiation of the cells and maintains their characteristic phenotype.

This is a picture of human proximal tubular cells treated with either--here's control cells, and this is at 24 hours, and generally cells are cuboidal. There are a few vesicles. But when you treat with either stauerosporin, which we use as a positive control for inducing apoptosis, or DCVC, and this is at 100, 200 and 500 micromolar, we see a marked increase in the number of intracellular vesicles, a change in the shape of the cells particularly at the higher concentrations and so-called apoptotic bodies. And we've even seen some changes at lower concentrations of DCVC as well.

So first, we compared just acute toxicity

or necrosis, I should say, looking over time courses of up to 48 hours in the primary cultures, and these were cells from males versus females, and in general, though, it's not a large difference, but there does appear to be a somewhat greater degree of acute toxicity from DCVC in cells from males than in females, which would be consistent with the rodent data.

Then, when we look at apoptosis, though, we have a markedly different dose and time dependence. I have the complete time course on the next slide. But here, one of the ways in which we measure apoptosis is by flow cytometry and FACS analysis, and cells are divided based on their phase of the cell cycle. We have resting cells, since this is--our exposures are typically done with confluent cells to more better mimic the in vivo kidney, which is generally nonproliferating.

So we have generally 70 to 80 percent in the G0/G1 phase; less, usually about 10 percent in the S phase, and the remainder in the G2/M phase,

and usually a fraction of a percent are subdiploid, and these values indicate the fraction that are subdiploid or apoptotic.

But when we treat with DCVC, for example, and this is 50 micromolar, so a fairly low dose, we see a large increase at 2 hours, even larger at 4, and it tends to drop down at later times. And I don't know how well this slide is going to show, but particularly in the back, I have to make it less complicated, but if you can follow the colors, what this shows is plotting out the same data for 2, 4, 8, 24, 48 hours, and the apoptotic cells are in the yellow bars, and what you see here, control in each one is a fraction of a percent over 48 hours.

Even at 10 micromolar DCVC, at 2 hours, we saw a significant increase from about 1 to an increase to about 5 percent, and this progresses where you go up to over 20 percent apoptotic cells. And the optimal response seems to be at about 4 hours and 50 micromolar. So these are indeed doses that are potentially relevant to probably high but

occupational or environmental exposures. And it tends to be--the response tends to be a fairly early, low dose response, because as the cells are exposed to higher doses, you get less apoptosis and at later times less.

And this is shown in a little different way, plotting here over time; the X axis is DCVC concentration, and this is percent of total apoptotic cells which is the circles, and we see again maximum levels generally at 50 to 100 micromolar, and the largest response is at 4 hours, and then, degree of the response tends to decrease.

But the other thing, and this sort of leads us into more of what we're pursuing now, is there's an indication that the cells can also undergo enhanced proliferation, because if you look at the percentage of S-phase cells, those tend to increase as well throughout time and also to be typically a more low-dose, earlier response.

And we corroborated this by measuring DNA synthesis in the cells with tritiated deoxythymidine triphosphate incorporation into DNA.

This is control. You see no change over 48 hours. Even with 10 micromolar DCVC, we see a significant increase, and this goes a little higher at 50 micromolar, 100, and then, of course, drops off because the cells are really less competent to be able to proliferate. So this is suggestive of some ability to proliferate.

How am I on time? I have no idea. Oh, okay. Thanks.

Okay; so, let's go back a second. So, and I'll get back to this in a moment, but this suggests that the cells, part of the response at low dose is that there can be enhanced proliferation, and I have a slide later on that indicates where we are going on that topic.

Next, I want to address the point of the potential role of beta-lyase and S-oxidase in necrosis and apoptosis in the human kidney. And what this slide summarizes, some data looking at acute injury and apoptosis. This is--the top panel is LDH release versus DCVC at either zero, the

control, 200 or 500 micromolar, and the cells are treated with either just buffer and DCVC, or they're pretreated with aminooxyacetic acid, which is an inhibitor of the beta-lyase or methimazole, which is a substrate for the FMO and competes.

And what is interesting is in the rat, I didn't show the data; that was published, actually, back in the eighties and the nineties, but in the rat, aminooxyacetic acid is very protective and provides very clear protection. And methimazole is moderately effective. In the human kidney, however, it seems to be a different story in that neither are very effective for acute toxicity, although methimazole produces some protection; a little less LDH release at 24 hours and a little more effective at 48 hours.

When we look at apoptosis, however, and here, again, note the difference in the time courses; when we look at necrosis, it's higher doses and a later time course, but with apoptosis, typically early times, 2 and 4 hours and 50 and 200 micromolar. We see here, for example, at 200

micromolar that aminooxyacetic acid here is marginally protective, but methimazole is significantly protective, and here at 4 hours aminooxyacetic acid didn't protect at all, whereas methimazole completely protected. So there is a difference both in the response, you know, depending on dose and the process one is looking at and there are species differences.

And the pathway specifically that we're talking about again is where trichloroethylene or TCE or tri either in the liver or the kidney is conjugated with glutathione to form DCVG, the glutathione conjugate, dichlorovinyl glutathione. And then, this all occurs in the kidney. It's processed by gamma-glutamyl transferase and dipeptase to form the cysteine conjugate, and then, it essentially has three fates: N-acetylation to form the mercapturate, N-acetyl DCVC, although this is--there's a deacetylase that can reverse this; beta-lyase or the FMO or S-oxidase, and both of these species are reactive electrophiles, and there's evidence of binding to DNA and to proteins

and certainly oxidative injury.

So this, again, is the pathway. And the sulfoxide is interesting in that it's actually a more potent toxicant in some respects than the cysteine conjugate. This shows again a picture of human proximal tubular cells treated for --these are primary cultures treated for 24 hours with DCVC sulfoxide, and you can --what was interesting here was that even at 10 micromolar, which is the top middle--I think my red light is going out here, but the top middle, you can see changes in terms of large increase in number of intracellular vesicles. Oh, yes. Well, okay, it's back, it seems to be.

You see a large increase in intracellular vesicles and strange changes in the shape of the cell. And then, this progresses at higher concentrations here; 50 micromolar, the cells are very elongated, large vesicles, apoptotic bodies. And this continues so that you barely have any recognizable cells, certainly by 500 micromolar, so just to illustrate what happens morphologically.

In terms of necrosis and apoptosis, fairly

similar to DCVC except that it's a little different. DCVC produces actually a little higher amounts of LDH release, and here, we see typically not until you get above 100 micromolar and 24 hours or later, you see the significant increases in what would be considered necrosis.

In terms of apoptosis, this is sort of a time course here, here's 0, 50, 100, 200, 500, and over time here, the controls are less than 1 percent. And you can see a progression with time and concentration; typically, but it seems to peak at earlier times and lower levels--I'm sorry, later times, more like 8 to 24 hours and at about 10 to 50 micromolar. So the pattern is somewhat different.

So, basically it would appear that in the rat kidney, the beta-lyase is more important. I didn't show any of that data. It was published more than a decade ago. But interestingly, the S-oxidase appears to be more important in the human kidney, and although humans seem to be overall less sensitive to DCVC than rats, the sulfoxide is a

more potent toxic species. And there are differences in terms of apoptosis and necrosis between DCVC and the sulfoxide in terms of which is the primary response.

So there are interesting, again, species differences and potential differences in the role of different bioactivating mechanisms.

So, to the last set of data I just wanted to briefly present was actually from a study that was supported by the EPA back in the mid to late nineties. We had a cooperative agreement. I was part of SERDP program, and this was actually, as I was telling someone earlier, that it was actually a case where the animal data followed up on the human data.

I didn't show this. I should have showed a summary slide of this, but we published a paper in 1999 demonstrating detection of dichlorovinyl glutathione in the blood of human volunteers exposed to trichloroethylene by inhalation. So we wanted to follow this up in experimental animals, because there, besides measuring blood and urine,

we could also, of course, measure tissues.

And so, male and female rats, and actually, I was telling someone earlier that now we mostly work with human kidneys, so we haven't done any work with rats in a number of years, actually since this study, but male and female rats were exposed to 2, 5 or 15 millimoles per kilogram of TCE or tri in corn oil by oral gavage. This was done to expose them to a low, moderately toxic and more highly toxic dose.

And then, we measured basically all of the metabolites: P450 and GST-derived metabolites in blood and urine at 24 and 48 hours and in liver and kidney homogenates at 2, 4, 8, 24, 48 hours and found some very interesting patterns, some of which I will try to summarize here.

This is showing formation of DCVG in rat blood, and it was found, interestingly, at the low does we actually saw in general more than at the higher doses over the time course, and the darker bars are female rats, the lighter bars are for male rats. And the time courses in general were very

irregular. We think that, certainly, enterohepatic circulation is coming into play. But what's interesting is that again, we see the glutathione conjugate in rat blood, and at the higher doses, we actually only saw it at certain times and in some cases only in are species or in one sex.

DCVC, which we could not detect in human kidney, we did detect at the highest dose in male rat blood. And it's shown here; ranges from about 8 to 12 picomoles per ml of blood. And this was not detected at the lower doses and was not detected in female rats.

So it was interesting that we could detect this metabolite as well in the blood, and it would make sense that it should be in the blood, because a lot of it will be formed in, for example, in the biliary tract and get back to the liver before getting back into the circulation.

Some other samples of data: in rat liver and tissue, we could detect the glutathione conjugate only in female rats in both cases, and there seemed to be a general peak between 4 and 8

hours, and then, it dropped, it disappeared, and then, we saw some again at 48 hours and much more in the liver than the kidney for most of the samples, but we had a couple of samples that were at very high levels and not in the male rat kidney or liver.

That was the glutathione conjugate. For the cysteine conjugate, you observe some at the medium and high dose, but here again, only in female rats for the medium dose and only in male rats for the high dose. And. you know, it's possible that they would have appeared if we had looked at additional times, but it's interesting that we can determine these intermediates, that there are species differences and gender differences.

And again, here DCVC in rat kidney was observed only in female rats, and again, it was increased from 2 to 4 hours then disappeared, and then, we saw again, presumably due to enterophepatic circulation. And then, in urine, actually, we recovered a large amount of DCVC in male rat urine

at all doses at 24 and 48 hours and only about, oh, less than 5 percent in the female rat urine.

Okay; so, that was from that study. Then, this slide which I will guide you through but it is just to kind of summarize sort of where we're going based on the data showing that there is enhanced proliferation. We know, for example, DCVC can cause oxidative stress and DNA damage under certain conditions. We have recently shown --I didn't bring any slides because of, you know, time limitations, but we showed, for example, in human kidney cells exposed to DCVC that we see increases in expression of heat shock protein 27 and p53.

Others have shown such as Grazyna Nowak in rabbit kidney, and in Europe, Bob van der Water's group had also shown changes in some of these signaling molecules. And these are known --for example, Hsp27 is known to alter cellular cytoskeleton and be involved in promoting cell growth and repair; also, Grazyna Nowak showed that protein kinase C was activated and again it's very dose-dependent. At higher doses you get more

toxicity. And additionally, there is evidence that the MAP kinase pathway is involved and then, you get signaling for changes in repair or apoptosis and growth arrest.

So this is kind of what we are currently working on in the human proximal tubular cells, and this is the direction we are going to try and look at the much more subtle effects and detailed mechanism of what determines the different potential responses in the human kidney.

So I want to acknowledge some of the people who worked on this over the years, different aspects: Brian Cummings was a graduate student of mine and then did a postdoctoral fellowship with Rick Schnellmann and is now an Assistant Professor at the University of Georgia. Dave Putt is my research assistant; Sarah Hueni was a research assistant; Poh-Gek Forkert at Queens University, we've worked together on some of the P450 data in the male reproductive system; Adnan Elfarra at the University of Wisconsin, we worked together on the sulfoxide story; and also support from the National

Institute of Environmental Health Sciences, grant support, and also, because I added at the last minute, I forgot to modify the sign, but the data on the in vivo exposure was supported by a cooperative agreement with the EPA from about four or five years ago.

And I'll be glad to answer any questions.

[Applause.]

DR. BUSSARD: There was a lot of data there, so let's see what questions we've got. Go ahead.

QUESTION: Janardan Pandey from Medical University in Charleston. Have you or to your knowledge anybody else has looked at whether the interindividual differences that you see in 2E1 levels may be because of the allelic variation at this locus. It's very polymorphic.

DR. LASH: I'm sorry; could you repeat, you said the interindividual differences?

QUESTION: Interindividual differences, they may be due to the allelic variation at this locus. It's a very polymorphic locus. Have you

looked into that or somebody else has looked into that to your knowledge?

DR. LASH: Not that I know of. I mean, we haven't looked into that. We've been, you know, just measuring in different, you know, samples. We had, as I said, some additional samples that provide more data in the same patterns, but we haven't looked into that, no.

QUESTION: John Lipscomb, USEPA. There are a number of issues with cytochrome P450 isoform, CYP2E1 in specific. It's important that when we remember polymorphisms that we remember the functionality of these polymorphisms and when and where and to what extent they are expressed in the genome becomes very important. There was a paper in Risk Analysis in December that I and some of the very wonderful people that I work with examined the impact of cytochrome P450 2E1 distribution among a population on trichloroethylene, so I would instruct some reading of that paper for your curiosity.

QUESTION: Larry, I've got a lot of

questions, but I'm only going to ask a couple. How's that?

DR. LASH: Okay.

QUESTION: The first one that piqued my interest was the one with dichloroacetic acid at very high levels in the testes without being at corresponding levels or higher levels of TCA. You got any explanation for that? It's reminiscent of some things we saw in blood is the reason I asked that question, obviously.

DR. LASH: Now you're referring, if I can get back to it--

QUESTION: Way back.

DR. LASH: Yes, I know.

QUESTION: That table where you had--

DR. LASH: Yes.

QUESTION: One case of TCA, but there's no relationship between TCA and DCA.

DR. LASH: Okay; yes.

QUESTION: And TCA has a much, much longer half life. So I'm curious why you'd have more.

DR. LASH: Yes, I mean, certainly in

analyzing the samples, we were cognizant of past problems with artifacts and concerns about accurately measuring it. And it only showed up in two samples--interestingly, this one sample subject number 7 also had a high level of TCA, but subject number 4, which had a high DCA, had no detectable TCA and had relatively low levels of the other metabolites.

QUESTION: That just doesn't fit with anything I've seen before. Okay; I'll let it go at that.

DR. LASH: I think that basically what one can just conclude from this is that, A, certainly, one can measure--it's another biomarker for exposure; you know, what it means and, you know, what the interindividual differences might mean is certainly an issue, but it's another means of--demonstrates that the metabolism occurs there.

Yes?

DR. SHIAO: In your cell culture experiment, you're talking about the proximal cell line. Is this a nontransformed or transformed

line?

DR. LASH: No, these are primary cultures.

DR. SHIAO: Primary cultures?

DR. LASH: Yes.

DR. SHIAO: This is tissue?

DR. LASH: So, I mean, rats, what we've done previously for rat proximal tubular cells, and we just isolate from the, you know, from the anesthetized rats, we take the kidneys. But from the human kidneys, we purchase, there is a nonprofit organization called International Bioresearch Solutions that they procure kidneys from hospitals and transplant centers, and typically, the kidneys are from accident victims or people who die of cardiac arrest, if their kidney is not usable, decreed to be not usable for transplant, and it's usually for reasons that are not important to us such as there's too much arterial plaque or sometimes a surgeon, I know in one case, they cut off the renal artery too close to the tissue, so there would be a problem in transplant or too much--you know,

excesses of glomerular sclerosis, for example, it's not going to influence our ability.

So the tissues are all normal, you know, pathologically normal, and we usually we get them within 24 hours of coming from the donor, so they're perfused with Wisconsin medium and kept on wet ice, and then, they're shipped out on the first plane. And then, you know, we isolate them within usually a few hours, grow them in culture.

So they're primaries. We don't passage them.

DR. SHIAO: And one of your slides showed, you exposed a different metabolite, TCE metabolite to the confluency. I'm wondering, this is the primary tissue, or this is the cell line?

DR. LASH: No, everything I showed was either some data I showed from freshly isolated cells, but mostly, it was from primary culture. For all the human data, it was all primary culture.

DR. SHIAO: And the FACS assays are using the cell lines?

DR. LASH: No the FACS analysis is

primary culture.

DR. SHIAO: Primary culture?

DR. LASH: Yes, primary, yes.

We've done some stuff with a rat kidney cell line for a totally different project, because for that one, we needed to use a stable cell line, because we're doing transfections to change expression levels of certain transport proteins. But in this case, we really want the primaries, because the problem with, you know, with epithelial cells in general and the proximal tubular cells in particular is that when you have a transformed cell or when you, say, passage them, they often, you know, they lose expression of many enzymes, particularly P450 and often GSTs.

They lose transporter functions, so their phenotype really changes. And we're interested in trying to define sort of the inherent susceptibility and factors involved, so you really need primaries. And using the serum-free, hormonally-defined media, which we tried to optimize with the addition of different growth

factors and hormones that we could maintain throughout the primary culture period, you know, high expression of mitochondrial enzymes and low expression of glycolytic enzymes, relatively high expression of brush border and P450s and GSTs and so forth.

So they're all primaries.

DR. SHIAO: Yes, but the reason I'm asking is because I'm wondering if the treatment of this TCE metabolite will induce a transformation in the--

DR. LASH: Well, that's another issue. I mean, the exposures are--for example, the apoptosis, we see as early as two hours. We have some data from one hour, but, you know, I don't think you're going to get that, you know, type of changes in this short time period. But we actually are planning on looking at--because I think that, based on our experience, I think those type of responses that are relevant, you know, to looking at transformation are going to occur in a longer time frame.

So the primaries, you need to passage them. And we've done some work with--in 2000, we had published some data where we passaged the human proximal tubular cells through six passages, and some, you know, some things, you know, P450s are bye-bye; I mean, they're gone. But the GSTs were still--some of them were there.

Some of the other functions, some functions were still retained. Morphologically, they looked fine, you know, so, it's sort of a model that we're trying to develop. But I think there's a lot of validation, and anything that you do or any type of conclusions you make from it has to be tempered by any changes in differentiation of the cells that occur as a consequence of--and not as a consequence of treatment.

But I think in the short time course of the experiments with the primary cultures, I don't think that issue comes up. I mean, we certainly have seen changes in expression of things like p53 and Hsp27 which, you know, may--and MAP kinases which may later on induce transformation, but

again, you know, those are short time courses.

QUESTION: Two quick questions; Scott, Dow. This data here actually, Dr. Forkert indicated that this is from the eight sterile subjects that she had.

DR. LASH: Right.

QUESTION: Have you been able to follow up with any nonsterile samples from subjects in the same environment?

DR. LASH: No, obviously, my role in this was the samples came from Canada; we were sent the samples to analyze. But, and I know, you know, obviously, it's not an easy thing to get, you know, the samples. So my understanding is she really could not get additional. But I think, you know, even though there is no paired control, we can still make conclusions about the fact that metabolites are detected; that there is variation; and that it is a potential biomarker.

And then, when we correlate that with showing P450 expression in tissue samples, it, you know, can fit together to that extent. But

unfortunately we haven't followed up.

QUESTION: Obviously, in the end result.

DR. LASH: Yes.

QUESTION: And the other thing is in the latest experiments that you showed your DCVC concentration in mice, did I read that correctly? Were those millimoles, 15 millimoles per kilo?

DR. LASH: They're micromoles. The in vitro? In the cell culture?

QUESTION: No, it looked like you were dosing animals and looking at the--

DR. LASH: Oh, you're talking about the--

QUESTION: Yes.

DR. LASH: The last ones. Now, that was--it was 2, 5 and 15 millimioles per kilogram is gavage--

QUESTION: Several thousand milligrams per kilo?

DR. LASH: I'm sorry? Yes, basically, in deciding on those doses, we actually used some of Dick's work. That was sort of a low moderately--a low toxic, moderately toxic and higher dose. And,

you know, obviously, to be able to detect some of the metabolites to you needed higher doses.

QUESTION: If it takes fairly high dose to detect the DCVC metabolite, I presume that's true, can you comment or do you have any data on whether, then, the in vitro concentrations observed are reflective of an in vivo situation, especially in a human?

DR. LASH: Well, yes, certainly what we have tried to do with the studies and the primary cultures of the human kidney cells is use as low doses as we can and, you know, in the past we and others have used millimolar concentrations for short-term responses, and those, really, are certainly not relevant, but we've--the doses we chose, we had 10 micromolar as our lowest dose.

And even at that dose, I think that--and again, this would be based on, you know, there's not a lot of good data to be able to specifically say that, you know, if you are exposed to, say, you

know, 100 parts per million, you know, in the environment, that you're going to have this dose in the kidney in this time frame. You know, it is a very dynamic process, as I think the time course in the rat showed.

But certainly, I think if you take some of the estimation from some of the modeling studies, which I presume we will hear about tomorrow from Dr. Bruckner and Fisher, that the 10 micromolar doses and even the 50, I think, is certainly within the realm of higher occupational and higher environmental exposures.

So I think those are relevant. And we do get subtle changes that seem to be reversible. I think once you get the higher, like, the 100, 200 micromolar, then, you are getting really beyond the bounds. But, you know, I think the 10 micromolar dose and even the 50 are relevant to the higher exposure, and if you talk about levels in drinking water in some of the, you know, instances where you're talking about, you know, parts per billion, then, that's, you know, orders of magnitude below

that.

But I think the 10 micromolar dose certainly has some relevance. You know, we have tried to get down as far as possible, and in some of our studies, which I didn't show, we've even gone down to 1 micromolar to look at and we've seen some small effects on expression of some of the signaling proteins. So we are attempting to address that.

QUESTION: From Ohio State. I have two questions. First, this one: it's quite interesting. Do you have the blood profile like this matched this exactly like this is from the same subject is my first question.

DR. LASH: No.

QUESTION: Second question is I noticed that somebody published or presented in the SOT meeting is the ovarian toxicity, so just one, did you measure any of that in the animal study?

DR. LASH: I'm sorry, what toxicity?

QUESTION: Ovarian, you know, from a female system.

DR. LASH: No, I'm not aware of that.

DR. BUSSARD: Let me just interrupt one moment, suggest that this be the last question, and then, we'll take a break, and then we may have time later to follow up.

QUESTION: Jeff Fisher, University of Georgia.

Larry, I have a question on what your opinion is about DCVC pathway and kidney tumors. Since the monograph came out in 2000, what's your opinion? Are there any other plausible hypotheses about the mode of action for kidney tumors that you think are relevant?

DR. LASH: Well, I think likely that one of the hypotheses that we had proposed was that you get these repeated exposure changes in, you know, mutations that cause changes in expression of certain regulatory proteins that affect cell growth and proliferation, and so, you get these repeated cycles of exposure, some injury and repair. And I think that's probably the most likely mechanism that really a nongenotoxic mechanism, because, you

know, the studies that have been done and that Martha Moore summarized in the EHP monograph, you know, really showed that the evidence is that DCVC is a genotoxin, but it's not very potent.

So that probably contributes to it, I think, to some extent but I think the likelihood is that, you know, some of the changes that we've observed, for example, you know, changes in p53, that in and of themselves, they probably are, you know, they're reversible changes and they're sort of a response-regulatory nature.

But I think when you have long-term exposures and all of these repeated instances of some injury and repair, that things eventually go awry as far as regulation of proliferation. I think that's probably the most likely. I think as far as any others, I don't know of any data that support, you know, any other hypotheses for mode of action.

QUESTION: Thank you.

[Applause.]

DR. BUSSARD: I want to thank everybody.

I think we'll take a 15 minute break, and we'll start back here at quarter to 3:00.

[Recess.]

DR. BUSSARD: We can start back up again and ask people to take seats. The next speaker is Richard Bull from MoBull Consulting. He's going to talk about TCE and liver tumors in mice.

DR. BULL: Good afternoon. Thanks for the invitation to participate in this. It seems like this happens about once or twice a year, and we go on again.

I should add that I've retired from both PNL and Washington State University, this last spring from Washington State. And I didn't do this work while I was at MoBull Consulting. This is work that's reflected back to mostly work that was done at PNL, Pacific Northwest National Lab, my former employers.

I was asked to review specifically these two papers, one, the contribution of dichloroacetate and trichloracetate to liver tumor induction by trichloroethylene and then some issues

about dichloroacetate or dichloroacetic acid, as it is often called, from trichloracetic acid by mouse in liver microsomes; it was something that was done with a graduate student some years ago.

I will have to make some reference to other things, because some of the things I'll be talking about come out of these other papers, and I just thought I would throw them in there, because even though I may not specifically quote them, some of the things that I'll talk about are in these papers rather than that paper.

And finally, there is a paper that we just got accepted in Toxicology that relates to interactions and the promoting activity of dichloroacetic acid and trichloracetic acid, I think will illuminate some of the things that we saw back in the 2002 paper that I'll try to get to in the end.

Essentially, the issue I think I was charged with is looking at DCA, the importance of DCA to the tumor responses; essentially, the contribution of trichloracetic acid to the liver

tumor response, I think, is goes back a long time, has been accepted, and the amount of DCA formed and how has become kind of a critical issue.

And most of what I will concentrate on will be looking at characteristics of the tumor responses as a way of trying to decide whether DCA is actually making a contribution, and I'll go very lightly over that and very quickly over that, because there's a lot to it, a lot of other work by other people that should be brought into this as well.

And then I will at least allude to this issue, if dichloroacetic acid is having a contribution, it has to be considered a low-dose contribution, not a high-dose contribution when you are talking about trichloroethylene, and so you have to be a little careful in extrapolating the dichloroacetic acid stuff directly to trichloracetic acid.

The implications of dichloroacetic acid contribution to liver tumors produced by trichloroethylene is, of course, trichloracetic

acid is a peroxisome proliferator, and to the extent people have tried, they have only been ab le to produce liver tumors in mice with trichloracetic acid. That happens to be consistent with happens with trichloroethylene, but that may be further reasons.

You have dichloroacetic acids making a contribution; it's clear that other mechanisms are involved. And it is, in fact, a multispecies carcinogen, and for those of us that have done work with it, it's kind of a nice thing to work with, because it will produce a lot of tumors in a fairly short period of time in a mouse.

I'm not going to spend any time on this. You've already seen the issue in terms of the oxidating metabolism of the oxidative metab olism of trichloroethylene, and there's others here that can talk about this in more detail than I will, but at issue really is the relative contribution of these to liver cancer, and the second issue is how do you get the dichloroacetic?

I've been asked to basically concentrate

on one paper that deals with this particular pathway, but in fact you'll see that I believe this to be a fairly small pathway and probably not the critical pathway as far as dichloroacetic acid formation from trichloroethylene.

This is some work--Hugh Barton is in the audience, so he can attest to how confident he in this particular data, but one of our big difficulties with looking at dichloroacetic acid and contribution to tumors in animals treated with trichloroethylene is it's there in very, very small concentrations, but you would predict that you would get this level of blood as an area under the curve if you did this at 0.5 grams of dichloroacetic acid in drinking water. It is clearly a carcinogenic dose.

However, the blood levels you see with the higher doses go up by a factor of more than 100 with just like a fourfold increase. So that high dose stuff is really hard to relate to anything that relates to trichloroethylene.

So we can get blood levels from

trichloroethylene roughly in the right area between 80 percent incidence and 0 percent incidence of dichloroacetic acid-induced tumors.

I'm going to talk a little bit about the one paper and leave this to questions if people want to ask more about it, because I'm not sure how critical an issue this is; maybe others will. We were really curious about it, because everybody was looking at trichloracetic acid as the source of the dichloroacetic acid. I don't think that fits with the other data anyway, but let's take a look at this.

My graduate student, Jim Urdank, was trying to look at this and couldn't figure out why he didn't see a radical form very clearly from trichloracetic acid; you get better formation from some of the other trihalogenated metabolites. And what he found, essentially, was that with the trap, you form the adduct, but it cytolizes, so you can't pick it up with ESR. And essentially, that's the name of the game. He just did the experiment and trapped, trapped the radical adduct and then

measured the trapped adduct by DC mass spec, and indeed, he found that it is there, and I don't think there is much question about it.

I think the big, more important question is how much. One of the other pieces of information that might be of interest as far as this particular proceeding is he did take a look at the production of this dichloroacetated radical from mice and rats in microsomes and in fact found that there was absolutely no difference in the amounts produced. It's produced by both rat and mouse microsomes.

Clearly, that is a problem, because we don't see the responses in the rat, so that is one issue to put aside.

Then, the issue, I think, you have to recognize is you can form that radical with any of the other metabolites; in fact, I think the folks at Wright-Patterson showed that this was, by far, the easiest to pick up, the radical that was produced in trichloroethanol; it was from either chloral hydrate or trichloracetic acid. So there

was nothing peculiar about the TCA being a source of the radical.

And the other problem that we deal with is regardless of which of these radicals is involved, you end up with the same end products in metabolism, so there is no way of looking downstream to figure out what's going on. You have to go back and take a look at each of these pathways if you're wanting to find where dichloroacetic acid might be coming from.

Irv Schultz, who published a paper here in 2002, did get some indirect evidence that you are seeing dichloroacetic acid formation in the administered trichloroethylene, and that's what this is showing. In essence, what the glutathione S-transferase zeta, for those of that don't know, is a dual functional enzyme. But it's inhibited fairly specifically by dichloroacetic acid but not trichloracetic acid or any of the other metabolites that we know of.

And so, Irv gave--I've forgotten the dose of trichloroethylene he gave here, I guess this is

here at 0.05. This would be in grams per kilogram, I believe; didn't note that when I grabbed the thing out of his paper, but this should be roughly in that range and looked at the level of the glutathione S-transferase zeta in the liver after that treatment; in fact, he did get a fairly substantial and significant reduction in the activity of that enzyme, and it doesn't prove the issue that dichloroacetic acid forming--it's consistent with the idea that dichloroacetic acid is formed with these trichloroethylene doses that have been associated with tumorigenesis in the NCI bioassay, et cetera.

So I think the conclusion that I would reach based on our experience with it is that TCA can be converted to dichloroacetic acid, but there are alternative pathways from dichloroacetic acid when you're administering trichloroethylene. To my knowledge, they haven't been quantified, at least not well. I am fairly certain that this does not account for it. Others have shown that trichloroethanol forms free radicals; it would

eventually give rise to dichloroacetic acid as well.

I think some of the work that Neil Pumford has done indicates that the dichloroacetyl chloride is formed from trichloroethylene, and that obviously could give rise to some dichloroacetic acid as well, and there may be other sources. I've heard some postulated. I don't know where that's gone from the DCVC would possibly get dichloroacetic acid formed, so there's a lot of ways for dichloroacetic acid to arise in the metabolism of trichloroethylene.

I mentioned that you do see the inhibition of glutathione S-transferase zeta with trichloroethylene administration, and that doesn't occur with TCA, at least.

The other issue, though, in hand, and this will be the topic of a second paper that I will review and some other things that go beyond that is that TCA produces a distinct phenotype of tumor. It's not distinct in the sense that other compounds are produced, but other peroxisome proliferators

will give you this same phenotype.

And if you saw significant conversion of TCA to DCA, the tumor phenotype should be observed with TCA treatment alone as well, and it is not. And that is the data I will be showing you later. So that's my bottom line is that you can form it, either as an adductor of dehalogenation. We have some other indications of the other pathways for trichloracetic acid degradation that don't go through DCA, so you can't even use the estimate of how much TCA you can't account for to come up with an idea of an upper bound.

So I hope that answers that question.

The other issue that I'll try to get back to this in a little more detail, but this is to illustrate what I'm talking about. We're trying to see how much of the liver tumor response that you might attribute to trichloroethylene from dichloroacetic acid. You have to really be quantitative. I mean, it's kind of funny to be talking about mechanisms that have, you know, demonstrated that, say, two grams per liter of

drinking water, which get very, very high doses; you notice this is a fourfold increase in dose, and you are seeing a peak concentration going up by a factor of 100.

And you only see this at night, and you only see this in high dose, because you are getting substrate inhibition of the metabolism, its own metabolism. It's a suicide inhibition that's been well demonstrated now.

And the levels that you're seeing with trichloroethylene we think you can kind of see will kind of fall down in this range below what you see with 0.5, which I've already shown you. So to talk about any dichloroacetic acid dose above the equivalent of this in drinking water or that equivalent in blood is really kind of hard to rationalize in the context of a contribution to trichloroethylene tumors.

Now, if we get down to this level, we see no tumors, but at that level, we're still seeing, like, an 80 percent tumor incidence in a lifetime.

The other issue that I'll talk to and I'll

try not to beat this to death because a lot of it is old data, and I'm sure those of you who are interested have read it, and those who haven't probably aren't interested, so, if you take a look at the liver of a mouse that's treated with dichloroacetic acid and trichloracetic acid, there's quite lot of difference, and they're not subtle differences.

One is with dichloroacetic acid-induced treated animals, you see these hepatocytes get very swollen, enlarged. They get enlarged with trichloracetic acid, but there's no comparison between these two. And what differentiates this from that is, in this case, you see quite a lot of accumulation of glycogen. This is, actually, if you look at it very carefully, you find decreases in glycogen, but you have to look at it locally, because you're getting a whole different distribution of glycogen, and it basically decreases glycogen.

The other thing that happens at high doses of dichloroacetic acid, we noted for a long time

and didn't understand what it was until some pathologist grabbed me and said what you're seeing is acid necroses. And you see it at a high dose of dichloroacetic acid, and I'm saying anything 2 grams per liter or higher, you will start seeing these kinds of lesions showing up.

We actually followed these with NMR, and they come and go, so you can't even depend on what you're seeing in the final analysis to tell you what you actually had. I've had experiments where I haven't seen it, but you could actually, with NMR, where you follow mice in time, and you can actually see these things come and go. The guy who was doing the NMR imaging for me was trying to figure out what the heck he was seeing.

But you don't really see it, we've never really seen it consistently at 0.5; the fact is I'm not sure we've ever seen it at 0.5 grams per liter, and we certainly don't see it at lower doses. So it's not necessarily a contributor at all to the low doses, but it could well contribute to the high dose effects you've seen with dichloroacetic acid.

So my argument is that this kind of pathology is an unlikely contributor to the trichloroethylene-induced tumors, and I've already mentioned the last point quite often. The paper that we published in 2002 depended on this particular observation, and that was that if we--there were other markers that could be used--Mike Pereira is in the audience; he could talk to this, because he's published extensively on other markers as well, but we chose to go at this c-Jun expression that we were measuring with the Santa Cruz antibody.

And what you see is within the tumors, and this is true all the way, is this staining for c-Jun. It turns out this is not c-Jun in the nucleus, this is actually a ubiquinated form of c-Jun that you can't pick up with all of the antibodies. You have to make sure you're looking at the antibody to the active site.

But you'll see that distribution there, and you'll see in the BUDR section that we took of the same in contiguous slides that you can see

where the c-Jun is high. That is where you are seeing the high levels of replication.

Now, this happens to be kind of what I would call a mixed phenotype tumor, where we see in both high staining and areas of low staining. This is what you see with the trichloracetic acid-induced tumor and when you look at c-Jun, and this is the tumor over here, and you can get a real good feel for where it's at because you can see the BUDR labeling down below. You see no indications of this accumulation of this ubiquinated c-Jun, essentially, in the cytosol of the cells.

So that's not uncommon with peroxisome proliferator-induced tumors, and that probably is suggestive that trichloracetic acid is primarily acting in the mice through that mechanism. The other thing is an old piece of data that I also want to point out that what happens, we did an experiment, it's basically a stop experiment where you induce tumors at a high dose and then came back and put the animals on varying levels of dichloroacetic acid in the drinking water to get

some idea what the cellular dynamics are within the tumor and the normal hepatocytes.

And two things are really clear. One is with chronic treatment, we're seeing really a substantial decrease in the replication rates within normal hepatocytes, maybe even seeing a little bit at 0.1 but certainly by 0.5 grams per liter. Now, it comes back up. This may be due to the acid or necrosis that we were talking about that you see at high levels; I don't know. There are some, maybe, other mechanisms we're kicking in.

The more interesting thing was, as you might have suspected from that last graph, those c-Jun stained areas are, as you get up to high doses, particularly, it's not significant until you get to 2 grams per liter. You're seeing a fairly substantial effect of DCA on the replication rates within the tumors.

And so, you get a little bit; you get this at a little bit lower dose and this at a higher dose. So there's kind of a complex interaction going on with the normal tissue in the tumor in

response to DCA treatment.

This just shows you--and I won't spend too much time on it--but you can actually replicate this phenotype by treating with dichloroacetic acid or trichloracetic acid, and cells, hepatocyte colonies derived from B-6 E through F-1 mice and put on soft agar. So you can actually produce the phenotype in vitro.

And rather than trying to cover this, and knowing that Mike was involved in four of these, and he's talking after me, I'll let him worry about that, but there are a variety of other differences that have been documented between dichloroacetic acid and trichloracetic acid-induced tumors.

This is more or less the data I was asked to speak to out of this particular paper, and essentially, what you are seeing is when you treat with these two doses of trichloracetic acid, you'll see uniformly what I'll call the c-Jun negative phenotype, whereas if you treat with dichloroacetic acid, we ended up with about a 50-50 split between c-Jun positive and c-Jun negative. That's a little

lower than we reported earlier, but this was also done by a different technician and a different histologist, so that might have contributed to it.

But clearly, we got a differing distribution. We got a fair number of c-Jun positive tumors with the dichloroacetic acid. Then, we did a mixed exposure of trichloracetic acid with varying doses of dichloroacetic acid to see what would happen, and what we found in this particular case is that we ended up with kind of a mixed phenotype as we increased the dichloroacetic acid dose. Didn't actually see any real clear-cut c-Jun positives, and this was kind of one of the pieces, open areas in the results.

But when we went up to the higher level, we started seeing a fairly high percentage of the mixed phenotype tumors, close to what we saw with dichloroacetic acid alone at both doses, I mean of the pure c-Jun phenotype, I should say.

And at the bottom, we gave 1 gram per kilogram body weight of trichloroethylene, and we ended up with a picture that at least is similar to

this one if not identical. We did find, in the case of the trichloroethylene-treated animals, we did actually end up with a fairly of strictly c-Jun positive lesions. But still, this bottom line is that this distribution is not compatible with the trichloracetic acid being the sole cause of those tumors.

I should add that the c-Jun positive phenotype is seen with almost every other type of carcinogen treatment except for peroxisome proliferators.

This is the dose-response information that came out of that particular, that same paper. It was a little bit atypical in the sense that what we ordinarily see with dichloroacetic acid treatment is something where we are at kind of a low response at this point in time and then a bigger response where we're seeing maybe 50 percent more tumors at that time point.

We didn't really use these out here as much except as a control, in essence. But if we score tumors per mouse, the one thing that we

found, at least when we started looking at the interactions of the low doses of dichloroacetic acid with high doses of trichloracetic acid, we did see something that looks like it's additive.

If you look at it, the additivity occurs here, but it's beginning to disappear already at that dose. This is--we had the mixed treatment. So there is near additivity at low doses. I'm not sure--you'll see it in a minute, because we've done another experiment that involved initiation promotion trying to get at the issue of how these two compounds might be interacting, partly for the interest of what might be going on with trichloroethylene.

So we used an initiation promotion protocol, where we initiated with vinyl carbamate at that dose, at 14 days of age, and at 21 days of age, we started administering carbon tet, dichloroacetic acid, trichloracetic acid alone or in combination beginning at 21 days. And then, we sacrificed animals basically at 18, 24, 30 and 36 weeks, and we had 10 animals per time period per

group, a total of 70 groups of 10 animals, considering that. And we had about 8,000 tumors, a little over 8,000 tumors scored.

We didn't diagnose them as to whether they're adenomas or carcinomas, because it was just too overwhelming to do it. We did look at a subset and found that we were identifying fairly accurately, so that was not a problem.

And the main thing we tried to do was we simply looked at tumor numbers versus tumor size, and I'll illustrate what you can see with this kind of experiment on this graph, and this happens to be the experiments with carbon tetrachloride. And we used carbon tetrachloride because it is more recognized as a cytotoxic agent that presumably promotes by virtue of cytotoxicity in a period of hyperplasia.

The interesting thing that you find when we look at tumor number, which would be, in most people's mind, an indication of tumor initiation in this kind of experiment, you get kind of a nice dose-response. And I had a lower dose and a higher

dose; just left them off, because it basically confirms the same thing, messes up the slide.

But if you start at the low doses, you start seeing some indications of promotion at about 20 milligrams per kilogram per day, and interestingly, you get up to 50 milligrams per kilogram, you get something that looks like a plateau. So it is like you flattened out, you maxed out the response, and it's pretty much a tumor the same number of tumors through the rest of the experiment.

If you go to higher doses, you get this. And if you get up to 500, it goes way up there. The tumor multiplicity just goes nuts, implying, as you get the higher doses of carbon tet, you're initiating probably through inflammatory processes, so it is kind of an interesting thing.

If you look at tumor volume, you find a very interesting thing, too, is when you take the low dose, it gives you an increase in size, just like you would hope it would. But then, if you go to the middle, the 50 milligram per kilogram dose,

you get really a nice increase in tumor size with time.

But if you go to the high dose, tumor size drops, so that really is very consistent with the idea that doses above 50 micrograms per kilogram really are initiating tumors as much or more than they are promoting tumors, which is kind of interesting, because the NCI bioassay I think if you recall was 500 and 1,000 milligrams per kilogram for carbon tet, so in this case, the promoting effects of carbon tet are much more interesting than the initiating events.

If you get to the paper, I'm showing you some data here that's not in the paper, because it just complicates it, and it's this time thing here. We basically did most of our comparisons between the 24th week of sacrifice and the 36th week of sacrifice, and here, I'm just showing for the DCA, two grams per liter alone, that's what we saw.

But what we're comparing here is the growth rate. This is tumor numbers, and this is the growth rate as calculated. And it turns out

you always have to have a couple of things that screw up your data. Here's one of them, and there's the other one, because you notice that that's occurring at the lowest dose of TCA, and I don't know if that's true or not.

There seems to be a general trend of inhibition of the tumor size with increasing doses of trichloracetic acid superimposed on a background dose of dichloroacetic acid, but it's noisy data. But it seems that it seems to be that there may be some inhibitory effects of trichloracetic acid.

What is really clear is when you put doses of dichloroacetic acid on the background of a high trichloracetic acid dose, and here's the same data where I plotted it out so you had all the time points, and what we're really comparing in the paper are what happens at 24 and 25 weeks. We actually had that particular dose group over time as well, which is actually the high dose with TCA and DCA. It gives a little better idea what the dynamics are.

But you'll see that you're coming up to

the same tumor number when you combine them and no cases that increase. But what you're really seeing is some changes in tumor number, perhaps not really consistently, that might reflect this. You're really inhibiting the growth rate of TCA-promoted tumors with dichloroacetic acid. So it's clear that these things aren't acting in the same fashion, and whatever is giving a selective advantage to one tumor type is not providing the same type of selective advantage to the other.

I don't know how I'm doing on time. I've got 12 minutes.

Getting back to this issue of the amounts of dichloroacetic acid and trichloracetic acid that are produced and what's realistically thought of in the context of contributing to the response that you've seen with trichloroethylene, what I've done here is taken from the pharmacokinetic studies, you know, some measure of the approximate blood concentration that one might expect and then compare that to the responses that one sees in a variety of circumstances. Liver tumors are induced

in this dose range; you get peroxisome proliferation in vivo. You get activation of the alpha receptor in that same region. You get hypomethylation somewhat in that same region; this is Mike's stuff. And then, the muogenesis stuff comes in at a quite a bit higher dose.

Now I will say there is one trick here, because when people only use one dose, I decided to just divide the no-el by the low-el, figuring that whatever happens in liver dose is not established yet, so that's a little mathematical thing I made to make this visual actually work, so I'll caution you as you go through the rest of these.

DCA, without going into the details, this is the tumorogenic dose range here in terms of blood levels, in terms of micromolar, and here's where you see--this will be about 0.5 in there. These are the variety of in vitro and I think even in vivo measures of mutagenic activity, which I won't go into the details. The kinds of blood levels you'd have to see to account in those studies that gave rise to those particular

responses.

Same thing here. This is the liver tumor range, and with dichloroacetic acid, here's peroxisome proliferator. It does cause peroxisome proliferation but not in the range that we're seeing tumors. It does have effects in vitro; it does activate the peroxisome proliferator receptor at some very high level, it's been shown. This is the work on hypomethylation, and Mike is going to talk about this; he may have some more doses; I don't know; this is just out of the literature some time ago.

And this, I just threw in, because dichloroacetic acid is known as an inhibitor of parv-a dehydrogenase kinase, and that occurs about in this range. That's the KI for dichloroacetic acid. So you're getting into a range of a lot of other things going on with interimmune metabolism when you get above those high dose ranges.

This data, there are some other things that have been reported in the literature, and a lot of this is by others, not by myself, by my own

lab, but here's the liver tumor range. Here is the range where you are starting to see suppressors of apoptosis, we see in our data the suppression of cell division in hepatocytes in approximately the same area.

We see stimulation of the cell division within. The tumors at a somewhat higher dose. We see, if we look at stimulated colony growth at one set of in vitro experiments, if we use naive animals, we see it takes quite a lot of dichloroacetic acid, but if you take the hepatocytes from a pretreated animal where you have inhibited the GST zeta, it takes quite a lot lower dose of dichloroacetic acid to produce the effect.

So we are not trying to determine the causes of tumors; if you are looking for the things that occur in the liver at doses where you see tumors, one of the things that we see is increased glycogen in this range. You get decreases in serum insulin. You get decreases in the insulin receptor in the normal hepatocytes, not in the tumor, and this is just in there for reference, for

biochemical effect.

So I conclude from this that the mixed phenotype of tumors induced by trichloroethylene indicates that both, to my mind, that both DCA and TCA probably contribute to the trichloroethylene tumors. DCA's contribution is a little more complicated, I think, than what we might have thought before, and that is the combined action. You have this later data says if you start getting substantial amounts of DCA, you are going to inhibit the development of the TCA phenotype. So it is not only DCA coming in and producing its own phenotype, it's also inhibiting the development of that, and I don't know how you untangle those.

So but the point is, I think, at low doses achieved from metabolism of trichloroethylene that this probably, I would guess, and that's not our data, the suppressed apoptosis may be more a more important mechanism than the stimulation of cell division.

And then, finally, you have to always come back and say why was the rat negative for liver

cancer? Easily explainable in part, because TCA is negative, and the rat makes plenty of TCA. But it may be simply that not enough dichloroacetic acid was formed with trichloroethylene to independently induce tumors, or perhaps it's DCA if it is formed is a better inhibitor; I don't know.

We've never really seen much in the way of DCA in a rat, so, except back in the days when we were generating artifacts.

This is to acknowledge--I won't go through these individually, and not all of them made contributions directly to what I'm reviewing here, but this is a list of coinvestigators and postdocs and graduate students that have been with me for a long time. Anja Stauber was really important, though, in the characterization of the phenotype. Jim Merdink is the one who did a lot of the metabolism work.

We were supported by a variety of agencies, if you will: Department of Energy supported some of this, Department of Defense, the SERDP program, USEPA, and indirectly, my NIH grant

contributed to it as well.

So with that, I will close and take any questions.

[Applause.]

QUESTION: Michael Pereira, Ohio State University.

Dick, on the vinyl carbamate study, I assume that was the hybrid mouse or--

DR. BULL: Yes, males.

QUESTION: Did you look at the lungs of those mice?

DR. BULL: No, we did not.

QUESTION: Because vinyl carbamate is a lung carcinogen and--

DR. BULL: It's a pretty good initiator in the liver as well.

QUESTION: Yes, but it's the standard--we use as the standard in chemoprevention, carcinogen to induce lung tumors in mice for chemoprevention studies. It's a very good one. It would be nice to see if you had the lungs.

DR. BULL: Well, we had all we could do

was count--get the liver tumors out and make sure we counted those accurately, so we didn't pay much attention to anything.

I would say, though, we didn't have any mortality along the way. We only lost a few animals except for those high carbon tetrachloride doses. We lost a lot of them early, but we lost very few animals in the DCA/TCA experiments. So if there was, it wasn't to the point it was pathologic.

QUESTION: Well, the lung tumors wouldn't have killed the mice. I mean, they could get nice big large carcinomas of the lungs with vinyl carbamate, and they still survived.

DR. BULL: Yes, that would have been interesting. I don't think we have the lungs, though. I would send them to you if we did.

[Laughter.]

DR. BUSSARD: Okay; let's move on to Mike Pereira from Ohio State University on carcinogenic mechanisms of TCE and its metabolites, DCA and TCA, DNA hypomethylation. And then, we'll see what

questions we've got before we wrap up. Thanks.

DR. PEREIRA: Thank you, and thank you for inviting me to speak here. I'm going to talk about some of our studies that are ongoing mainly wit h DCA and TCA with respect to inducing DNA hypomethylation.

There's two types of alteration, I'm sure most of you know this, but I'll go very rapidly through this. There are basically two types of alteration of DNA methylation found during carcinogenesis. One is the hypomethylation of DNA and the other is the hypermethylation of tumor suppressor genes. DNA hypomethylation is found early in all solid tumors; it can be found even in normal appearing tissues and precancerous lesions, where there's about a 30 to 60 percent decrease in the total extent of DNA methylation.

DNA methylation is important because it decreases the binding of methyl DNA binding proteins. This alters the binding and the recruitment of transcription factors and enzymes involved in histone modification, especially histone

deacetylase, this increases the acetylation of histones, which increases the expression of genes.

DNA hypomethylation also results in chromosomal instability, and ironically, it does result in the hypermethylation of tumor suppression genes. Here's a cartoon looking at DNA methylation in normal tissue. Normally, you have the methylation sites recruiting methyl binding proteins, there's at least six different methyl binding proteins. This recruits HDAC, histone deacetylates that deacetylates histones and turns off genes.

In tumors, you see DNA hypomethylation, a decrease in the binding of the methyl binding protein, a decrease in HDAC, and you get acetylation of histones, and this turns on various genes. Actually, DNA hypomethylation doesn't turn on genes,what it does, it allows the genes to be turned on by

transcription factors, et cetera.

Okay; the other alteration in methylation is the hypermethylation of tumor suppression genes. These are methylation in CPG islands of exon 1 and the upstream promoter that are unmethylated in normal tissue, and they become hypermethylated in tumors. They're usually found in the later stage than DNA hypomethylation, although in certain tumors, you can actually find hypermethylation of tumor suppressor genes in normal appearing tissues. And it's a major mechanism for –down-regulating the mRNA expression of tumor suppressor genes.

Yesterday, I actually gave the opposite talk to NCI in which I talked about cancer prevention and the reversal of DNA hypomethylation and hypermethylation. And this is a cartoon showing that in the normal tissue, you don't have methylation in these islands; you have acetylation of histones; in the tumor you get the methyl binding protein, HDAC

recruitment, deacetylation and methylation with histones that turn off these genes.

Okay; I'm going to go over and present some of our data of the hypomethylation that is induced by DCA, TCA and TCE in normal tissue and in tumors. What we have shown is that DCA, TCA and TCE induce hypomethylation within days in the liver; other nongenotoxic carcinogens, including the trihalomethanes, peroxisome proliferators, phenobarbital, they all induce DNA hypomethylation within days.

I'm going to show some data that the liver tumors that are promoted or induced by DCA and TCA contain DNA hypomethylation; that upon cessation of exposure, the DNA hypomethylation in DCA-induced tumors goes away, whereas the DNA hypomethylation in TCA-promoted tumors is not reversible, and it's correlated with what we have found and Dick Bull and we have found that DCA tumors regress upon cessation of exposure, whereas TCA tumors do not.

We had shown that methionine prevents, as well as not only prevents but actually reverses the

DNA hypomethylation induced by TCA, DCA trichloroethylene and peroxisome proliferators. We also shown that chloroform prevents DCA but not TCA-induced DNA hypomethylation and that methionine and chloroform both prevent DCA-induced liver tumors and foci and that chloroform does not prevent TCA-induced liver tumors; thus, tumor prevention is limited to the methionine-chloroform, the correlation of their effect on DNA hypomethylation.

Chloroform increases DCA but not TCA-induced DNA hypomethylation in mouse kidney and that chloroform increases DCA but not TCA-promoted kidney tumors. We've also shown that DNA hypomethylations a good marker, for both route of administration and for cancer chemoprevention and that DNA hypomethylation induced by these agents decreases the extent to which histones are acetylated.

And I'll give you some of that data. But before I give the data, let me just go back into how this fits into the mechanism of TCA, DCA and TCE carcinogenic activity. They all induce DNA

hypomethylation. And this results in a field cancerization, that is, there is an increased risk of cancer due to the accomplishments of one of the first alterations required for cancer to develop, that is, DNA hypomethylation is now present; you don't have to have it.

This allows precancerous cells or whatever you want to call them, or cells at risk, to now have chromosome instability occur, to have hypermethylation of their tumor suppressor genes and progress on to cancer. And that's what we're saying here.

This forward effect allows for an increase in histone acetylation and binding of transcription factors that increases the expression of protooncogenes. This also results in the hypermethylation of tumor suppressor genes. You have to have the hypomethylation first before you're going to have the hypermethylation of these tumor suppressor genes unless it is a hereditary event.

And it also gives you the chromosome

instability or ace breaks, rearrangements and exchanges. That all goes on to the progression towards cancer.

The first thing I want to talk about is the DNA hypomethylation in DCA and TCA-induced mouse liver tumors. In this situation, let me go back one, we measured DNA methylation in this case by HPLC.

And you can see in the noninvolved liver from DCA-treated animals, the methylation is normal, the same as in naive mice. In the adenomas, the methylation is decreased, and if you removed the DCA for 21 days, methylation goes back up. As I mentioned, that correlates with other studies by Dick Bull and myself showing that these adenomas that are promoter or induced by DCA will regress.

When you look at the TCA tumors, we did

not see regression. Again, DNA methylation in the noninvolved liver from a TCA-treated animal is the same as the control; the adenomas are lower, recovery, for 21 days, is low; carcinomas are a little bit lower, and recovery is low . So the TCA-treated tumors, we did not see the reversal of DNA hypomethylation that we did see in the DCA-treated tumors, which corresponds to the regression in DCA but not TCA.

Some of our other studies in the correlation between the ability of DCA and TCA to induce DNA hypomethylation and their ability to induce liver tumors. In this study, what we looked at, we had shown that methionine both prevents and reverses DCA and TCA-induced DNA hypomethylation. So we wanted to see is that correlated with the prevention of liver tumors.

First, I'll just give you a little bit of the data we have, in which we have shown that DCA, TCA and TCE all induce DNA hypomethylation. In this situation, we measured DNA methylation by looking at CCGG sites and the ability of HEPA-2

to cut those sites when they are not methylated. When they are methylated, they are resistant to the restriction enzyme, and it doesn't cut the DNA, and plus, you don't see any bands. And that's why you don't see any bands in the control.

And when you have the CCGG sites become unmethylated, the restriction enzyme can now cut it, and you now see bands. And you as you can see, DCA without the methionine is zero; TCA and TCE all cause DNA hypomethylation of these genes, and methionine appeared to prevent the hypomethylation. These are different doses of methionine: zero, 30, 100 and 300 milligrams per kilogram.

And we have quite a view, and some of those publication have more data about showing how the reversal occurs and a little bit maybe into some of the mechanism, how the methionine prevents and reverses DNA hypomethylation. But based on these studies, we decided to see whether methionine would now prevent the DCA-induced liver tumors.

In this study, we gave the

mice, 3.2 grams per liter of DCA in the drinking water and 0, 4 and 8 grams per kilogram of methionine in the diet. We had two sacrifices, one to see the effect in normal liver and the other one to see whether or not there was an effect on tumors.

This is the response of foci in adenomas, and as we see here, methionine prevented the adenomas induced by DCA quite extensively, and the foci were prevented at the higher dose but not the lower dose, which suggests that methionine slows the progression of foci through adenomas. This is what we see with most chemopreventive agents that prevent cancer, that they actually act by slowing the progression, and that's probably why we had a buildup although not statistically significant increase in foci at the lower dose.

We looked at liver to body weight ratio, and the methionine had no effect on the liver to body weight ratio. The increase in liver weight was not effected by methionine. Here's DCA, low dose, high dose methionine, TCA low dose, high dose

methionine. No effect.

We looked at peroxisome proliferation. Here's the DCA, low dose methionine, high dose methionine, no effect of methionine on peroxisome proliferation induced by DCA.

We looked at glycogen accumulation. And we did see a little bit of an effect of methionine, although it's still very high compared to normal. There appeared to be a limited effect of methionine on DCA-induced glycogen accumulation. In this study, we looked at the DNA methylation by using a monoclonal antibody that's specific for 5-methocytidine. And all I'm showing here is that 5-methylcytidine competes with the antibody showing specificity since cytidine does not compete with the antibody for the DNA, showing that the antibody is specific for methylated cytidine.

And then, when we looked at DNA hypomethylation using this procedure, we did see an increase with methionine in the methylation. But in the liver, DCA caused DNA hypomethylation, and methionine prevented the

hypomethylation.

The conclusions from this study are summarized here, basically, that methionine can prevent DCA-induced DNA hypomethylation and that it can prevent DCA-induced liver tumors. Methionine did not prevent DCA-induced increase in liver weight, peroxisome proliferation and had only a relatively limited effect on glycogen accumulation and that the methionine appeared to slow progression of foci to adenomas, classical chemopreventive agent.

We also did another study looking at chloroform's effect on the carcinogenic activity of DCA and TCA, because the three of them are found together in drinking water. Here are some of the parameters we measured, and I'm going to present some of the data that we have.

This is the protocol. This is not the tumor data; actually, this is the study we did before that, in which we want to see the effect of chloroform on DCA and TCA-induced DNA hypomethylation. So we put the animal in chloroform at 0, 400, 800 and 1,600 milligrams per

liter, and then at 13, 14, 15, et cetera days, we gave them DCA and TCA by oral gavage at 500 milligrams per kilogram.

This shows that chloroform, yes, does induce DNA hypomethylation when given in drinking water at 800 and 1,600 but not at 400. We have had a much more extensive dose-response curve done with chloroform, and it does appear that somewhere between 500 to 800 is where you break in the ability of chloroform to cause DNA hypomethylation.

And here is the chloroform in the presence of DCA.
As you can see, chloroform does a good job of preventing DCA-induced DNA hypomethylation. And this is a curve showing that, looking at the three different bands, that as you increase chloroform, the amount of DNA hypomethylation decreases.

When we looked at TCA in the presence of chloroform, we saw no effect of chloroform on TCA-induced DNA

hypomethylation, and here are the band scan and averages of the experiment showing that there is no effect of chloroform on TCA-induced DNA hypomethylation. This summarizes that.

So, after that study, we decided, then, to see what chloroform would do on TCA and DCA-induced liver tumors. We gave chloroform in the drinking water, and with either TCA or DCA, we started at seven weeks, and sacrificed at 52 weeks, and you see here, there was no effect, as we saw with the methionine, there was no effect of chloroform at either 800 or 1,600 milligrams per liter on DCA or TCA-induced increase in liver weight.

However, when we looked at the tumors, chloroform does a very good job of preventing DCA-induced foci in female mice, You don't see foci induced by TCA. That's one of the differences you see between DCA and TCA-induced liver tumors. You don't see foci

with TCA, very hard to find. With DCA, you do see a lot of foci, just like with DCA, you see a lot of adenomas but very few carcinomas. With TCA you see carcinomas but not as many adenomas.

When we looked at the tumors in the female mice, again, DCA was prevented by chloroform with no statistically significant effects, on TCA tumors. At the high dosage use of chloroform, there was a statistically significant decrease in foci induced by DCA; again, very few foci with TCA. Looking at the tumors, where DCA, again, chloroform prevented the high dose DCA tumors but did not affect the TCA tumors.

Okay; and we also looked at the kidney, and in this study, we did find kidney tumors promoted by TCA but not significantly promoted by DCA. However, in the presence of chloroform, we did get a significant increase in DCA individual kidney tumors with high doses of chloroform. There was no effect of chloroform on TCA induced tumors in the kidneys.

In summary, this study is basically that chloroform prevented the carcinogenic activity of

DCA but not TCA in the liver, and this correlated with its prevention of DCA but not TCA induced DNA hypomethylation. Again, we find a good correlation between DNA hypomethylation and the effect of a preventive agent on tumor yield.

Chloroform did not effect the kidney tumors promoted by TCA, while it did enhance the activity of DCA-promoted tumors. And in the kidney, I didn't have the data, but chloroform enhanced DCA but not TCA-induced DNA hypomethylation. And that also correlated with enhancement of DCA but not TCA-promoted kidney tumors. Again, demonstrating a correlation between DNA hypomethylation and tumor promotion.

This is one study we did, trichloromethane, basically; just looking at route of administration, and I'll just sort of give the bottom line of it is that we gave chloroform by gavage, and we also gave it in the drinking water. And when we looked at DNA hypomethylation, looking at band three, which is just one of the bands, we could have taken any one of the bands from those; they all get the same

curve, you get a nice curve by giving chloroform by gavage, but you give the same dose, the animals getting the same dose in the drinking water, the ability to induce DNA hypomethylation is much lower, corresponding to the decreased carcinogenic activity of chloroform when given in drinking water relative to its carcinogenic activity by gavage.

Okay; the other thing we looked at is the hypermethylation of tumor suppressor genes induced by DCA and TCA. We looked at the estrogen receptor alpha and p16. Basically, we chose these two genes because we're looking at colon tumors and lung tumors in the mouse, and they're very good biomarkers, the hypermethylation of these genes for cancer prevention in the colon and the lung.

And in this, what we did is we'd isolate the DNA. We'd treat the isolated DNA with bisulfate. Unmethylated DNA is converted to uracil, methylated cytosine are resistant and remain cytosine. You

the bisulfated Dna is cloned and PCR the methylated cytosines remain as cytosine; the unmethylated cytosines are copied as thymine so that you're able to determine which cytosines are methylatied.

And this is our results with the estrogen receptor alpha looking at exon 1 and a little bit upstream from it, and you can see in normal liver, very few methylation sites; the same in DCA-treated non-involved liver, but in the DCA-induced liver tumors, you have quite extensive amount of DNA methylation of the estrogen receptor alpha.

And the same thing, of course, in the TCA-induced liver tumors. You also get an increase in the methylation of the estrogen receptor alpha. This is data that was done I don't know how many years ago and I kind of forgot about it, but I put it together, and we haven't actually pursued this, but we are doing it now. We are looking at the expression of the MRNA, since the methylation should turn it off.

Hopefully, he will get the mRNA done and maybe publish the data.

And we also looked at p16, as I mentioned, and there's the normal liver, the noninvolved; there is some methylation, and there is some in the tumors but not a significant increase. And the same appears to be true with the TCA, although there might be a little bit more, but it doesn't look as impressive as the estrogen receptor alpha

Going back to one of my first slides about the mechanism of the DNA hypomethylation, again, I'd just like to mention that it's a field cancerization with the increased risk and the increased expression of protooncogenes in the DNA hypomethylation, chromosome stability, and that could be the mechanism how DCA and TCA and TCE are promoting liver tumors.

Thank you.

[Applause.]

QUESTION: Hi, thank you very much. Larry Moore from the Medical University of South Carolina

at Charleston. With respect to c-mc hypomethylation in your studies, is c-mc hypomethylated to the same extent or in the same way in humans, specifically with respect to human hepatocytes, as it is in the mice that you've looked at? There's something different going on between mice and humans here.

DR. PEREIRA: You're getting at whether it's affecting expression, et cetera.

QUESTION: Right, exactly.

DR. PEREIRA: It's only used as a biomarker. If you ever write in a paper, and you put in the paper the c-mc expression, the paper will not be published, because it has very little if anything to do with the expression of c-mc. We're only using it as a biomarker for DNA hypomethylation. You could have picked any gene, and you're going to find hypomethylation. We've done insulin-like growth factor-II; you could even take a tumor suppressor gene like the estrogen receptor alpha and find other areas that are hypomethylated.

So it has nothing really to do with the

expression. The only reason we used it is because the HPLC procedure was not sensitive enough in the tumors to measure DNA hypomethylation. I mean, I've had numerous papers turned back when they talk about expression, and when we had the expression, it does correlate, but you can pick any one of probably 1,000 or 2,000 genes, because if you're going to have basically a 50 percent decrease in DNA methylation, it's going to effect all of your genes.

QUESTION: Yes, I think I have a followup question to Larry's question; Jeff Fisher. Has anyone done gene array or protein arrays to look at the relationship between hypomethylation and to get ideas on what's important in terms of transcription?

DR. PEREIRA: You have to get--and that's partly because some people don't--you have to get away completely from the idea. This has nothing at all to do with expression of these genes. It has

only with the fact that it allows expression to occur, because we've done other studies, in which I could give trichloroethylene, and you give it and give it. In three days; you get DNA hypomethylation. You then give it again, and you get a peak of expression, and it decreases. You give it a dose again, it peaks and decreases. It only allows the expression of the gene. And you can look at c-mc, jun, h-ras and IGF-2. It does not in itself mean that you're going to get expression.

But you see in tumors a whole array of genes that are increased, these tumors, these protooncogen es. And that ability to have that occur is related to the fact that you're getting this hypomethylation that opens up the chromatin, I didn't get the data with the histone acetylation, because you're getting acetylation of histones that is required for expression, and that allows, then, transcription factors and other factors to come in and allows you to get expression.

If you start talking about--my whole talk yesterday for and I actually started at 3:00 and ended at about a quarter to 5:00, was on DNA hypomethylation, and I didn't once talk about expression, because it was on prevention. And, I mean, expression does change, but it's not a direct effect hypomethylation.

This is the most--actually, if you ask me, the critical thing about DNA hypermethylation, because you're not going to get hypermethylation of tumor suppressor genes until you get DNA hypomethylation. And the reason is--it all goes back to my first slide here. And the people who talk about the expression really do a disservice. It all goes back to this slide here.

If you change this to a tumor suppressor gene, and in this situation, and it's methylated here, near that area, it's going to be compressed. You can't get the hypermethylation until you open it up, and you get this situation, when it becomes hypermethylated. Because remember, tumors have an increase of DNA methyltransferase. So they have all

the machinery required to methylate the DNA. And once you increase this, you then get your hypermethylation, and that allows, since it gives the cells an advantage to grow, to cone out, and that's where you see tumor suppressor genes hypermethylated and not the other genes.

QUESTION: Thank you.

DR. PEREIRA: I'm sorry.

Go ahead.

QUESTION: Hi, I'm Jennifer Sass with the Natural Resources Defense Council here in Washington. Thanks for the very interesting talk. I thought it was a nice followup to Dick's talk as well, and I'm going to ask you a question that tries to make sense of both of them together.

As I understand what you're saying, you're saying that the DNA hypomethylation for trichloroethylene and also TCA and DCA would predict a risk for tumors in the animal systems that you have been using, and it would do so in a way that doesn't involve peroxisome proliferation; is that right?

DR. PEREIRA: Yes, exactly, has nothing to do with that.

QUESTION: Which Dick in his talk said could, I mean, he sort of referenced your coming and said that, you know, he was open to that as well. So I thought that was a nice followup.

And what I want to ask second is do you think that because your work has stuck with the animal models, but obviously, you're going to the NCI and presenting cancer prevention type hypotheses, so you must be pretty confident that what you're showing is also relevant to the human situation; is that right?

DR. PEREIRA: Right; in humans, they look DNA hypomethylation as a biomarker in chemopreventive studies. And actually, I have those studies ongoing. We have one ongoing in China and another ongoing at Ohio State University. But to go back to your one question with the proliferation, we did a study with the Wyeth compound, and we did partial hepatectomy. The idea was to determine the relationship between cell Proliferation and DNA hypomethylation.

So we wanted to see if we induced cell proliferation, maybe that would speed it up the hypomethylation. And it doesn't. So that's not really related to cell proliferation.

QUESTION: Thank you.

DR. PEREIRA: It's more likely related to the effect of these agents on proteins that bind DNA and on opening up the chromatin.

DR. BUSSARD: Are there any more questions?

[No response.]

DR. BUSSARD: Okay; I think we're done. Thank you very much. I want to thank all the speakers. We'll start at 8:00 tomorrow morning, and have a good evening and a good dinner, and we'll see you in the morning. Thanks.

[Whereupon, at 3:59 p.m., the meeting recessed, to reconvene at 8:00 a.m., Friday, February 27, 2004.]