FOOD AND DRUG ADMINISTRATION

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CENTER FOR DRUG EVALUATION AND RESEARCH

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ADVISORY COMMITTEE FOR PHARMACEUTICAL SCIENCE

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MEETING

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THURSDAY,

MARCH 9, 2000

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The meeting was held at 8:30 a.m. in the Advisory Committee Conference Room, 5630 Fishers Lane, Rockville, Maryland, Dr. John Doull, Chairman, presiding.

PRESENT:

JOHN DOULL, M.D., Ph.D.	Chairman
GLORIA ANDERSON, Ph.D.	Consumer Representative
JOY CAVAGNARO, Ph.D.	Industry Representative
JACK H. DEAN, Ph.D.	Industry Representative
DAVID M. ESSAYAN, M.D.	FDA Representative
JAY GOODMAN, Ph.D.	Academic Representative
JAMES MACGREGOR, Ph.D.	FDA Representative
RAYMOND TENNANT, Ph.D.	NIH Representative
IGOR CERNY, Pharm.D.	Executive Secretary

A-G-E-N-D-A

A-G-E-N-D-A (Cont'd)

P-R-O-C-E-E-D-I-N-G-S

(8:36 a.m.)

CHAIRMAN DOULL: Allow me to welcome you all to our Subcommittee, the Non-Clinical Subcommittee. We are part of the advisory committee for Pharmaceutical Sciences, and we are meeting this morning. I think the agenda is out on the table.

Let me introduce the members of the Subcommittee. Let me start over there with Dr. Goodman. Jay, why don't you introduce yourself.

DR. GOODMAN: My name is Jay Goodman. I am a Professor of Pharmacology and Toxicology at Michigan State University.

CHAIRMAN DOULL: Joy?

DR. CAVAGNARO: My name is Joy Cavagnaro, and I am President of Access Bio, representing Bio companies.

DR. DEAN: I'm Jack Dean. I am with the Sanofi-Synthelabo Pharmaceuticals, and I am responsible for preclinical development.

CHAIRMAN DOULL: I am John Doull from KU Med. Gloria?

DR. ANDERSON: I am Gloria Anderson, Callaway Professor of Chemistry at Morris Brown College in Atlanta.

DR. MacGREGOR: I'm Jim MacGregor. I am Director of the Office of Testing and Research in the Center for Drug Evaluation and Research, and I am the principle FDA coordinator for the Subcommittee.

CHAIRMAN DOULL: I might just mention that I understand we have a new Subcommittee member or will have very shortly. It will be Dr. Ray Tennant from NIEHS. And Ray is with us this morning. Kimberly has changed jobs and we have a new Exec. Why don't you introduce yourself.

DR. CERNY: Sure. My name is Igor Cerny, and I am taking over for Kimberly Topper this meeting. I am going to read the conflict of interest statement.

CHAIRMAN DOULL: Okay.

DR. CERNY: The following announcement addresses conflict of interest with regard to this meeting and is made a part of the record to preclude even the appearance of such at this meeting.

In accordance with 18 USC 208, general

matters waivers have been granted to all committee participants who have interests in companies or organizations which could be affected by the committee's discussions of collaborative approaches of the scientific research issues of common interest to the pharmaceutical industry, universities, the public and FDA.

Specific areas of focus will be in the non-clinical studies areas of interspecies biomarkers of toxicity and non-invasive imaging. A copy of these waiver statements may be obtained by submitting a written request to the Agency's Freedom of Information Office, Room 12A30, Parklawn Building.

In the event the discussions involve any other participants or firms not already on the agenda for which an FDA participant has a financial interest, the participants are aware of the need to exclude themselves from such involvement, and their exclusion will be noted for the record.

With respect to all other participants, we ask in the interest of fairness that they address any current or previous financial involvement with any

firm whose product they may wish to comment upon.

I would like to sort of warn the audience that the committee is going to attempt to work through lunch. There is a lunch scheduled for 12:30 today. And that we will honor the break at 10:30. But at 12:30, when you think it is all going to stop, it is not going to stop. People will probably keep talking through that time. So you may want to run out during the break and get some food. The committee, I understand, will be brought in food. They will apparently be paying $6.00 a head, and the transcriber is our treat, because they have to work through lunch.

We have out there this Website information for how to get the slides from this meeting and the transcript from the dockets. That will be available on the Web about three weeks after the meeting. So that information is out there as well as the docket number for this meeting that we will be using. So as I said, we will warn you again about this lunch situation at break so that you can run out and try to get something to eat.

CHAIRMAN DOULL: Rest rooms are right

outside. The telephone is down the hall. Do we have any other business? Oh, I might say one word about the minutes. The minutes have been prepared from our last meeting and have been circulated, I think, to the committee. If you will look at those during the meeting, towards the end of the meeting we can I think formally approve the minutes. Any other things, Jim?

Well, I think we might as well go ahead and start. We have a very busy schedule. As Igor has said, we are going to try and work through lunch and keep up with the schedule. So let me introduce again Dr. Jim MacGregor from Food and Drug.

DR. MacGREGOR: Thanks, John. As I said, I am the FDA coordinator for this Subcommittee, and I thought we would just begin the day with a very brief review of the objectives and focus of the Subcommittee and FDA's expectations for today's agenda.

This Subcommittee was created as a means for the FDA to obtain advice and improve scientific approaches to regulating non-clinical drug development. And in addition to that traditional role, which the oversight committee or advisory

committee for Pharmaceutical Science has been providing for some time, we have also charged this committee with taking an active role in fostering scientific collaborations among FDA, industry, academia and the public to pursue and facilitate objectives that come out of the recommendations that are made by the Subcommittee.

Now just a little more specifically, the objectives are envisioned that the committee will be recommending approaches and mechanisms to improve the non-clinical information for effective drug development, to improve the predictivity of non-clinical tests for human outcomes, and to improve the linkage between non-clinical and clinical studies. And then as I have said, in addition to providing advice and recommendations in these areas to actually facilitate collaborative approaches to advancing the science and regulation of drug development.

Now the role of this Subcommittee is to identify and recommend focus areas in the non-clinical science where FDA should be focusing its resources. And then to foster the gathering of advice and

facilitation of collaborations through identifying experts in these focus areas and forming expert working groups in those areas.

The process by which these expert groups would be formed would be an open public process that would involve announcements in the Federal Register, solicitation of recommendations from the collaborators and members of the committee, FDA stakeholders from industry, academia and the public, and by going to appropriate professional societies in the disciplinary areas involved in the focus areas and asking them to recommend the best experts in those disciplines to participate.

Then these expert working groups are envisioned as providing specific recommendations and facilitating collaborative work to gather information that is needed.

The Subcommittee, this Subcommittee, is envisioned as a steering committee to these expert groups and to the collaborative projects that might be implemented by these expert groups. And that this subcommittee would also support the output from those

working groups by supporting facilitating workshops and reports, et cetera, that would come from the expert groups and indeed might decide to do their own as well.

Now at the last meeting, which was in December, the Subcommittee in fact did narrow the focus and recommend that initial focus should be on two principle areas, and that was biomarkers of toxicity and non-invasive technologies to link non-clinical and clinical studies.

So the focus of today's meeting is to identify within these focus areas more specifically how we should move forward to implement these objectives. And in order to do that, we have structured this meeting around these two areas, and we have asked two representatives from the FDA who are leading programs, who are leading the current FDA programs in these areas, to organize sessions around these topics to give their own perspective from the FDA perspective in these areas, and then to invite in experts from these areas to discuss the state of the science, opportunities, and to give collaborator

perspectives on approaching these two areas.

So the two people that have put together the focus areas are Frank Sistare, who is Director of the Division of Applied Pharmacology Research in CDER, who has put together the biomarker part, and Dr. Jerry Collins is Director of the Laboratory of Clinical Pharmacology in CDER, who has put together the imaging part.

Our hope is that by the end of the day, if possible, that the Subcommittee will be able to provide recommendations to the FDA on the program and focus that is presented today, and that the Subcommittee will consider the formation of expert working groups to develop more specific recommendations and to begin to facilitate collaborations in the proposed areas.

So those are our expectations. As John said, it is a very full day. I would like to thank the committee in advance for agreeing to try to jam as much as we have into a single day, and I hope and am looking forward to a very exciting and productive day.

Okay, let me just introduce Frank Sistare,

who is going to present the FDA perspective and summary of our program and recommendations in the imaging area and coordinate the other speakers in this area.

DR. SISTARE: Okay. As Jim pointed out, we are going to start off the session on biomarkers of toxicity. The key that we are focusing in on here is that these biomarkers be accessible. Accessible so that we can move from animals into the clinic.

The ultimate goal of what we are trying to achieve here is to establish a more optimal set of easily accessible biomarkers allowing progression from animal studies into the clinic and actually through the clinic and potentially at the patient bedside and eventually be adopted into the practice of medicine. These biomarkers should herald the early onset of drug toxicity, obviously prior to any morbidity or any irreversibility.

The impact if this whole project area is successful -- the impacts could be many. We could assess better the relevance or irrelevance of animal toxicity findings. We could accurately assess those

doses that are associated with toxicity, maximize favorable impact on public health, minimize the many regulatory dilemmas we get into when we don't have ways to assess the relevance or irrelevance of animal findings and the impasses in drug-hold situations that we get into with our sponsors. To improve selection of candidates for drug development and reduce candidate attrition rates. And to accelerate drug development and potentially minimize resource consumption.

So in this morning's set of presentations, we are going to hear from some of the technology leaders in this field and how they are impacting the field. What they are doing and what can be done that we are not yet doing. We are going to hear from leaders in the field of 2-D gel proteomics, Gordon Holt from Oxford Glycosciences and Leigh Anderson from Large Scale Proteomics. Although I think unless Leigh's voice doesn't improve, we may hear from Sandra Steiner instead. So he has got a back-up over here. I think we will be hearing from Sandra instead. Leigh has come down with a case of laryngitis unfortunately.

Chip Petricoin will be talking about another technology to discover new proteins that may be good biomarkers -- surface enhanced laser dissorption ionization of SELDI Technology. Then we are going to hear from Spencer Farr from Phase 1 and Frederico Goodsaid from PE Biosystems, who are going to talk about technologies that can take advantage of looking at signals, gene expression signals in peripheral blood cells. Again, the thinking being that we can move from animals into man with accessible biomarkers.

But before we move into some of these areas of the technology providers, we are going to hear an overview from Greg Downing on the NIH perspective on biomarkers, and we are going to hear from Malcolm York from Glaxo Wellcome, who is going to talk very specifically about a couple of biomarkers that Glaxo Wellcome has developed a lot of very nice data on. And the thinking being here that maybe what we need to do is to start -- as Glaxo Wellcome is showing, start with a couple of biomarkers and get those into a level of acceptance that we can all agree

to. Get the level of anxiety down in terms of what these biomarkers can and cannot do for us. And we are going to propose actually that some of these biomarkers -- and some of you may have heard some of our earlier presentations last time -- that we have also developed an interest in troponin as a cardiotoxicity biomarker and that this may be a very fruitful area to start and a good place to do some quick learning.

So I am going to ask the speakers to just come up here. I have essentially introduced them all. They will reintroduce themselves. We are going to start with Greg. There is sort of a tight schedule and I will warn you that Dr. Doull can be very ruthless, so please stick to the allotted 20 or 25 minutes. Fortunately, we are a little bit ahead of schedule. So, Dr. Downing.

DR. DOWNING: Well, good morning. I hope everyone is enjoying early springtime activities here in Bethesda. It seems like just a couple of weeks ago we were sitting here under two feet of snow.

I am here from the Office of Science

Policy in the Directors Office of NIH, and I have talked to a number of groups that have involved you. So if you guys all know this already, you can sort of sit back and read the paper or whatever. I appreciate the opportunity to talk with this group. The NIH is very interested in this initiative and the two topics on your working group's activities are ones that are near and dear to NIH's research interest these days.

I would like to again mention that Ray Tennant from the National Institutes of Environmental Health Sciences is part of this committee, and we are very glad that NIH is represented in such a role. This represents, I think, a focal point for a number of institutes' and centers' interest in toxicity biomarkers.

I would like to briefly share some of our experiences over the last two years. This is really an initiative that Dr. Harold Varmus, when he was Director at NIH, initiated, and the momentum is now focused primarily in the institutes and centers, although the Acting Director, Dr. Ruth Kirschstein, is again very interested in this activity. And we think

we have had some significant progress over the past year since an international conference was held on this particular topic.

I would like to share a brief overview of where this is going and I think share some of the experiences from other models that we have developed at NIH, and then end with some common points and challenges that working together and fostering new research in this particular area brings to light.

Why this became an issue for NIH I think should be probably self evident. But there are a number of major issues that membership from industry and academic health centers and others brought to the attention of Dr. Varmus approximately two years ago now that there is an emerging bottleneck in drug discovery and that through genomics and combinatorial chemistry and other high throughput mechanisms that there are a lot more things to test. And the challenge is to try to do this in an efficient fashion and try to speed the transition from basic discoveries to clinical therapies to help public health basically.

So the speeding in the translation of

basic sciences is focused on developing new tools and applying them in a clinical setting and understanding the disease processes through the use of fundamental biology and the new emerging tools of technology. Obviously, the near completion of the human genome is going to present a great deal of opportunities to explore new therapies and focusing on a variety of different markers for diseases is one of the focuses in trying to improve the efficiency of the drug development process.

These are a brief overview of some of the NIH activities over the past two years. The one I would like to focus on is the April of 1999 Multidisciplinary Conference in which there were several sessions that I think are quite relevant to the discussions of this committee. There is a specific initiative that National Institute of Environmental Health Sciences has been advocating and sponsoring the research activities in, and this started with a meeting held in November of 1999 with interest from industry and academia, building upon the experiences of the April 1999 conference.

Throughout this talk, there will be a series of Websites that I encourage people to visit that provide a variety of different perspectives on NIH research activities in this area. There are likely others, and one of our major underlying goals at NIH is to try to enhance the understanding and identify to the research community the needs for biomarkers in a variety of different settings and early stages of drug development as well as in clinical trials. There are copies of the handouts floating around and I will post the talk today on our Website that is listed here.

I think it is helpful to start with some definitions. This was identified to us as a major issue early on in this process that the terms used in discussing the use of biological parameters and evaluating the effects of drugs on human systems -- there are a variety of terms used in the literature. And Dr. Varmus commissioned a working group that involved leadership from industry and FDA and academic centers to come up with some common terms and a conceptual model for the use of markers in the drug evaluation process.

I just would like to suggest that this has been adopted pretty much through a variety of organizations now, this definition listed here, that a biomarker is any characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. And this term is meant to be broad in its interpretation in that biomarkers can be structural, genetic proteins, physiologic processes that one can detect with imaging technologies, and includes biobehavioral type measures that are used in evaluating various pharmacologic therapies.

Now one aspect of this is the use of markers in decision points regarding new therapies and their evaluation in clinical trials. And these are also definitions that are not new but have been incorporated by this working group as places to focus on what the meaning and application of markers might be. There has been a substantial interest in the use of markers in substitutions for clinical endpoints in clinical trials, and we felt that it is important to

state these definitions for encouraging common use of language. The clinical endpoint is a characteristic or variable that reflects how a patient feels, functions or survives. A substitute endpoint or a surrogate is a biomarker intended to substitute for a clinical endpoint. And a surrogate endpoint is expected to predict a clinical benefit or harm or the lack of benefit or harm based on epidemiologic and therapeutic and pathophysiologic or other scientific evidence.

I will not go into the details of all of this today, but the focus of NIH's interest has not been necessarily on developing new surrogate endpoints, but developing the scientific framework in which biomarkers may be incorporated in the evaluation of therapies in clinical trials.

This is a conceptual model that was actually shown here back last September I believe and one of the committee members spent some time discussing this. But the point here is to show a progression of how markers may be applied in a variety of settings in decision making about new therapies.

The point to be made here is that the committee recognized very early on that the importance of biomarkers in evaluating toxicity is an important factor. We do not -- the committee did not feel strongly that the biomarkers for toxicity could substitute for clinical toxicology endpoints. However, they could be very helpful in informing the provisional evaluation of efficacy and toxicity and judgments being made about potential surrogate endpoints.

I won't go through the processes here. We hope that -- a more definitive paper is currently in review and will hopefully provide greater insights into how the evidence is built to provide that biomarkers may be used in this particular process. The underlying theme here is that the use of markers for toxicity assessment is recognized as to be an important component of what biological parameters can be used for in the assessment of novel therapies.

This is just a list of the various aspects of how biomarkers can contribute to the development of these therapies. And the focus has really been on the

early phases of this and serve many functions from the industry side of drug development. And this is actually a place where we feel that NIH research can probably make the greatest impact initially. And basically the predevelopment studies in helping correlate the diagnosis and prognosis of disease with specific biological parameters of disease -- identifying specific proteins or genes that are related to these -- and investigating pathophysiologic mechanisms of disease. Biomarkers by their discovery can provide a great deal of insight in driving hypothesis-derived research and determining a greater understanding of disease processes.

I preclinical studies, biomarkers can be used often in the confirmation of in vivo activity and in helping explore the concentration response relationships, and these can often help glean and determine the best candidates for further clinical trials.

In Phase I and Phase II studies, these help evaluate activity and develop dose response relationships. And in Phase III clinical studies, one

area where we are starting to see some great interest is the use of markers in the stratification of study populations. By having more homogeneous groups, it reduces the numbers and the power and the size of the groups needed to adequately power studies. And that by defining groups by certain biological factors or parameters in their disease process, one can reduce the numbers of subjects that are needed in Phase III clinical trials.

These markers can also be useful in an interim analysis of efficacy and safety and applied towards regulatory approval, which I won't dwell on today. And I think an important factor that Frank mentioned earlier is that the use of these markers ultimately can be helpful in the decision making that actually occurs in clinics and in healthcare settings. These are also helpful in identifying who responds to therapy and who doesn't rather than waiting for a clinical endpoint to be achieved. And also very helpful in prognosticating and predicting disease measures, and this is of great public health interest today.

At the spring conference last year, there were two groups that I think are directly related to this particular discussion today, and they led to some discussions about key research gaps. And Frank was a part of this committee as a number of speakers here today were also involved. And I just wanted to point out some of the key issues that were identified as needs in biomarkers for toxicity and safety assessment in this group. They are listed here, but basically that there is felt to be a variety of markers that are not being adequately utilized in the research community by evaluating clinical responses or in the early phases of drug development. It is also felt that there is an awareness gap that the applications of the tools and technologies that are employed in the basic science research laboratories are not reaching their full utility in evaluating therapies in patients. There is a great deal of work that needs to be done before relying upon these, but it is felt that the full extent of the application of these new technologies and tools such as the gene arrays, proteomics imaging technologies, the fundamental tools

of discovery in the laboratories, are not being fully achieved in terms of their appropriate use in clinical trials and assessment of clinical responses.

It is also felt that there is a need to develop data associating particular genes, proteins and small molecules to human pathology. Coordinated efforts to relate animal toxicity markers to human responses is needed. And that there is a need for key organ system toxicity markers to be developed further.

The group felt that the best opportunities for developing toxicity markers were related to the understanding of the alterations in signal transduction and other pathways such as inflammatory markers to clinical toxicity for particular agents. Case studies and having key examples of these were helpful in aiming the discussions in these working groups.

It is also felt that defining genes related to human toxicology is important. I will take a moment to address a working group two years ago at NIH who focused and developed a successful new pharmacogenomics initiative. Identifying the

development of toxicogenomics as a field was a high priority for them and encouraged other institutes and organizations to invest in their scientific framework for developing toxicologic markers.

And then it is also felt that there is an opportunity now for the development of high throughput screening methods to correlate gene expression with protein expression as they relate to toxicologic responses in drugs.

The use of emerging technologies -- and I am somewhat hesitant to use these as emerging technologies anymore as they are becoming quite widespread in application and their uses. But nevertheless, it is important to focus that the development and application of analytical tools to discover small molecules was felt to be important in assessing drug toxicity. We have heard a lot about the use of GC and mass spec for this particular purpose, and the utilization of imaging technologies to understand toxicologic mechanisms at molecular, whole organ and whole body levels.

NIH is -- you will hear more about this

this afternoon -- is making major strides in both the basic and the applied fields of imaging technologies. In fact, this year the office -- in the Director's Office, the Office of Bioengineering and Bioimaging has been formed, and this is going to, I think, spearhead trans-NIH efforts in applying new applications of imaging technologies.

Encouraged expansion of the application of cDNA array technologies and proteomics. You will hear more about that today. And in the clinical technologies, it is felt that there is a need for less invasive tools. And one of the institutes, the National Institute of Dental and Craniofacial disorders, has a specific interest in developing technologies to utilize salivary secretions and other clinical measuring tools as more appropriate and less invasive tools in measuring various drug effects and biological parameters.

And then as another emphasis, the use of humanized transgenic animal models to evaluate absorption, distribution, metabolism and excretion as well as the molecular and tissue and organ-specific

toxicologic effects.

There are some infrastructure needs that were pointed out, and it was felt that there is a need to stimulate collaboration amongst the sectors of the biomedical research enterprise to develop and evaluate toxicologic markers. We actually find this to be true throughout NIH in that there is not a specific institute that is focused primarily on toxicity of drugs, but this is something that is spread throughout the institutes. And trying to bring together collaborative research organizations or programs throughout NIH is a challenge for us, and we look to find new targets of activity that would bridge various areas of science and be useful to this committee as well as in the therapeutic development.

It is felt that one suggestion was to establish a consortium of public and private institutions to achieve these goals and to utilize funding strategies and networks for markers evaluation that are similar to the NCI models that I will discuss briefly at the end of this.

In the past nine months, a number of

initiatives are underway or being planned for institution this fiscal year, and two of these I would like to highlight, as they involve collaborations in a public/private collaboration. That being the Cancer Biomarkers Laboratories, and many of you are already aware of that. These are a number of just a sampling of some of the research initiatives that are underway this particular year that I think will contribute to the development of biomarkers. And the pharmacogenomics that I mentioned earlier I think provides some of the framework for pulling laboratories together to achieve data bases on unique responses that individuals have in regards to drug mechanisms.

A neuro-informatics initiative was underway this year to develop an infrastructure for which imaging technologies can be pulled together and analyzed across different types of imaging modalities. The osteoarthritis initiative I will just say a few words about. There is interest in diabetes markers from a variety of institutes in the focus of our research discovery programs. Imaging programs in

neurologic diseases and immunomodulatory markers, particularly for transplantation rejection, are underway. Cardiovascular markers and new programs in hematologic disorders and toxicity markers, as I mentioned earlier, as well as new programs in chronic lung disease.

There are a variety of models that we are trying to experiment with to see what works best and applying them in other institutes and disease areas. One of these is the cancer biomarkers area that Dr. Klausner initiated approximately a year and a half ago. It was to develop an infrastructure to start from the basics in evaluating molecular and genetic and other biomarkers in early cancer detection and risk assessment.

There are three components to this. One is the discovery markers, the second is the technology development, and the third, the clinical validation of relationships of markers in the population. This is a very complex organization but works streamline to interface biotech, the academic laboratories and industry to streamline the development in a systematic

way of various candidate markers. Currently, these are focused on solid tumors for ovarian, prostate, lung, liver and colon and breast cancer. These have been quite successful in the six months since the discovery laboratories have been brought on line. This spring, the technology development centers will be activated, soon to be followed by the clinical validation.

And there has been widespread interest in this from the applications, and we expect that this program will continue to grow. The Website listed for this has a great deal of information now about the kinds of markers that are being evaluated, and I encourage people to activate that.

The osteoarthritis initiative is a public/private partnership that is being developed with industry in advocacy organizations in NIH to develop new infrastructure to conduct prospective natural history cohorts, in evaluating both imaging and biochemical and genetic markers for this degenerative disease. This is a model based on interest from the community of a large need for

markers in a disease that has no disease modifying therapies currently is felt to build on the advances in imaging of joints and musculoskeletal structures. This will hopefully be underway later this year and will utilize a variety of different technologies and again will be done as a partnership involving academic and biotechnology laboratories.

The next step from an NIH perspective in a broad sense is addressing some of the informatics needs for this. It was pointed out in a number of points during our travels through the biomarker arena that the organization of the literature and the use of terminology and the classification of markers is at best scattered. And that one cannot necessarily trace the development of a marker from its discovery in a laboratory to its applications in various settings in the clinic. And through that -- by identifying this as a need, NIH has explored several options for developing data bases or informatics systems. In fact, data bases have been recommended and attempted in several institutes in disease-specific settings and have not worked very well because of a high number of

issues, including the high amount of maintenance that is required for this.

So actually we have spent about six months exploring various options, and I think the place where we are going now is to develop Web-based knowledge centers that through Internet technology, we can link various data bases and publications and data sets that can be categorized in a variety of different fashions. The importance of this is to make this user friendly in that the information content be of value to the user. And we have explored and have arrangements with the various organizations now to try to use Internet technology to bridge these data sets that are in various places and allow people to do analyses of various data sets in a much more easier fashion than pulling together various articles.

A process doing this for one specific disease area and pulling together just the last five years of information took us approximately three months of one person's time and developing sort of a composite of the information was a very laborious process. And our goal is to try to achieve this in a

fashion that utilizes the current Pubmed system as well as analyzing or access to various data bases that contain such information.

In terms of resource development, a number of these programs have developed new specimen and image repositories and assay validation laboratories. We are working on now developing Websites that will contain information on various specimen repositories that will help the research community very quickly identify appropriate samples for which they can submit research proposals and utilize these samples for their analyses. NIH has had a longstanding history in the development of epidemiologic and longitudinal data bases which are important in relating changes in biomarkers over time with their clinical outcomes.

There is a further need to expand and extend the laboratory technology for clinical measures and improve the accuracy and precision of these tools, many of these you will be hearing about today. And there is quite an expressed need in the clinical research realm for the technical expertise in expanding the clinical research capacity to use these

tools and understanding how they interact with particular disease processes. And throughout this, we have understood that there is a need for integration of new disciplines to bring people with new skills to the field and understanding the clinical toxicologic manifestations of how biomarkers and therapeutic interactions can be interpreted in a clinical setting.

I think that from my perspective this is a unique opportunity to bring some of the science together and identify some of the major challenges in toxicologic markers for various needs in drug development. We think -- we are eager to hear your recommendations of areas of need and if possible to prioritize them. And from our vantage point, our goal at least in the Director's Office at NIH is to try to bring together research communities and to leverage resources and achieve useful scientific technologies to foster this development. I would be happy to take any questions if there are any. If not, thank you very much. Jim?

DR. MacGREGOR: Not a question. I would just like to make a comment that I think that NIH

participation in this effort is absolutely critical. We went to NIH and asked them to select for us the appropriate representative for the committee, and Ray Tennant was selected because our initial focus area is the area of safety, toxicity, biomarkers and NIEHS has been designated the lead agency for that. But Greg has graciously indicated his interest in this and has said that he will continue to participate and as necessary, he will serve in place of Ray if Ray can't make it to the meetings. Anyway, I am extremely pleased to have NIH added to the committee. I guess we have not made that official yet, and I apologize to Ray Tennant. But I guess I would ask our advisors and consultants and our chair that if we can, even if Ray's appointment hasn't become official, I would hope that we can include him in the discussions today. Because I think NIH participation and inclusion is critical to our success.

CHAIRMAN DOULL: Why don't we move on then and hear from Malcolm York from Glaxo. Can somebody back there catch the lights?

MR. YORK: I thought bringing slides would

make things easier. Obviously not. Well, thank you for the opportunity to come over to present to the Subcommittee. Basically what I am going to present is part of a presentation I gave in London, which was on establishing surrogate markers of safety and efficacy, which Frank Sistare was also at. And he felt that some of the case study material that I presented would provide a useful contribution to this Subcommittee hearing. So I hope that proves to be the case.

DR. DEAN: Question, Mr. Chairman.

CHAIRMAN DOULL: Yes?

DR. DEAN: Do we have the slides for this presentation? A copy of the slides?

CHAIRMAN DOULL: Not for the --

MR. YORK: No. I will forward those electronically. The two cases studies that I will present this morning will be, one, looking at cardiac troponin I as a measurement in myocardial damage in the Wister Han rat and the Marmoset. And the second -- so basically using a human diagnostics immunoassay for troponin I and applying that to nonclinical species. The second case study will be looking at

utilization of the SELDI protein chip technology and biomarker identification in skeletal muscle toxicity, again in the Wister Han rat.

So the troponin. Basically troponin's protein complex regulate the contraction striated muscle, and there are three subunits within the complex, C, T and I. And in the clinical arena, they have been used to reflect myocardial damage, degradation of the contractile actin-troponin complex, causing release of the troponin molecules into the bloodstream and that can be a reflection either of severe ischemia or cell necrosis.

Troponin I has three different isoforms -- slow twitch skeletal muscle, fast twitch skeletal muscle, and the cardiac muscle, troponin I. Troponin T -- cardiac troponin T -- is also found in the cardiac muscle. Predominantly the data I will present today will be on troponin I.

All these three isoforms are encoded by three different genes. The cardiac troponin I molecule has an additional 31 amino acids at the end terminus, which gives it its unique identity.

The feature that is very important in terms of applying this original clinical biomarker to nonclinical species is that the molecular itself is highly conserved across the mammalian species. And this was a feature that we looked at to build on.

The troponin I data is a two-site sandwich immunoassay. And I won't go through the main features as I say, but just to point out the antibodies involved. The first antibody is a goat polyclonal anti-troponin I molecule linked to the acridinium ester. And the second antibody is two mouse monoclonal antibodies. So there are three antibodies within the assay procedure. Basically, they are linked to paramagnetic particles. So what will happen is they will, following the reaction antigen-antibody binding, the complex will remain captured by paramagnetic particles that fluctuate in solids, and then the relative light units release will be proportional to the concentration of troponin I in the molecule or in the sample rather.

In terms of initial validation, it was, again, a very crude procedure. Basically, we took

hearts from a dog, Marmosets, rats, and also from the mouse, and just basically did serodilutions of heart hemogenates to look for cross reactivity. And very good parallelism curves were obtained for the dog, rat and for the Marmoset. But with the mouse, there was confounding cross-reactivity which maybe wasn't unexpected given the presence of mouse antibodies in the assay reagent.

One of the interesting things in looking at cardiac troponin I was the identification by cardiac histopathology of acute myocardial necrosis with a compound that is in exploratory development. The mechanism was thought to be due to reflex tachycardia following an acute dose of the compound. The tachycardia was felt to cause ischemia leading to the myocardia damage. So one of the pathologists basically put forward this proposal and designed the study. There were four groups of animals. A control group dosed with saline; group two, propranolol using it as a beta blocker agent; group three, propranolol and the cardiotoxin; and then group four was the cardiotoxin only. There are individual groups with

each time point. So we collected samples and hearts at 6, 12, 24 and 48 hours.

In terms of the measures carried out, we looked at the troponin I and then looked at troponin T later on. And then the more traditional measures of cardiac toxicity -- AST, CK, LD, isoenzymes. And we also had a look at CK-MB mass of myoglobin in the rat, again by automated immunochemoluminesence. But we could find no cross reactivity with these procedures. And hence, there is no data generated on these.

Histologic examination of the hearts was performed, and also we looked at the stability of the troponin I molecule in terms of storage. In terms of the data that I will subsequently present, the traditional markers showed no change between control and treated animals, and I will show the histology representative histology slide later on.

This is troponin results in the animals which were group four -- group four only. So it did appear as though the proposed mechanism of toxicity did occur through the beta receptors. And here I just want to point out the increase in troponin molecules.

We have got troponin I and troponin T. And increases above the sensitivity -- the sensitivity of the assay was .03 micrograms per liter. And these increases were seen in five out of the six animals at six hours and predominantly three out of six animals at twelve hours. But by 24 hours and 48 hours, generally the troponin increases have disappeared. So in terms of its marker, very clearly it was up at 6 hours and 12 hours, but then started to be eliminated from the plasma. And this gave good concordance with its appearance in the clinical sample in the human. Following damage, the half-life at first appearance is between 6 and 12 hours. So there is good correlation it seems there.

In terms of the pathology, there is no pathology seen at six hours, and the first signs of pathology -- I'll move on to the slide in a minute -- came at 12 hours, 24 hours and 48 hours. So certainly the troponin I molecule was showing the first signs of myocardial necrosis ahead of histopathology.

This is a representative slide taken from one of the group four animals at 24 hours. The green

arrow indicates normal areas of the heart. The blue arrow indicates damaged areas of the heart. So here we have areas of myodegeneration -- focal areas of myodegeneration present in inflammatory cells, and these are occurring in the subendocardial region.

In terms of its stability, I will come on to talk about stability in more depth later one. But this is troponin I and this is looked at. So this is original data which was measured within a few hours of sample collection. And then we have had another look at 20 weeks later -- sorry, 12 weeks later and 24 weeks later. So you can see that the results start to disappear or the positivity starts to decrease. But these are still positive results. Although it is very small increases in respect of the total rise of troponin I. For example, if you had severe myocardial infarction in the human, you would expect to see values maybe 25 to 50 micrograms per liter. So this appears to be a very sensitive and correlating with very minor degrees of myocardial damage observed in the heart.

In terms of its application to other

species, this is data -- again, this was retrospective examination. The pathologies to identified areas of myocardial damage in the Marmoset study, 7-day dosing study. And we basically went back to those samples collected for toxico-kinetic analysis and measured troponin I. And this again represents the data from two animals with cardiac pathology. The day zero, the values are below sensitivity. At zero hours, below the sensitivity of the assay at .15. By four hours and eight hours, there is a clear increase in troponin I seen in the Marmoset. And by 24 hours on day zero, the values had not returned to normal. These are clear positive results.

If you look at day six -- again, sort of looking at a chronic parameter, there are still increases seen. But with this particular animal, it does appear to clear within that 24-hour time period.

Again, with the Marmosets, we measured CK-MB mass by automated immunochemoluminescence. And again we found very good data or correlation between these two markers in terms of the change. But in terms of false sensitivity, the troponin I molecule

seemed to provide the best data.

In terms of its specificity, I said earlier that in terms of checking its specificity that there were different isoforms of troponin I that also occurred in skeletal muscle. How would this be occurring in a toxicological situation where skeletal muscle toxicity was observed? So here we had examples from a 7-day pipeline study, so 7 doses. And skeletal muscle toxicity was observed. It was felt that this toxicity started to occur around about day 2 or day 3 of dosing. These samples are just collected at day 7. This is the control data here, and this is the group three data.

Protein kinase in the rat has a very short half-life of time points of an hour to two hours. So the increases seen here, and some of them are no increases, are not actually going to be in concord with the degree with skeletal muscle damage. The better markers are AST in this respect, even though it is a ubiquitous enzyme. But correlating the two, we had -- and also again with the histopathology, which showed damage to the intercostal muscles in this study

in the Wister rat.

So we had again a retrospective look at troponin I and could find five out of the six animals with no change in troponin I and showing a specificity. In the one animal which showed an increase, we went back and could find evidence of cardiac toxicity.

In terms of the current limitations of the troponin I assay, again this is very much from the clinical endpoint, is stability of the molecule. Troponin I does undergo in vitro proteolysis. And clearly the selection of the antibodies is quite important. There is a stable region between 30 and 110 amino acids. But the actual region that confers its specificity, the third 2L amino acid region, is actually very -- is actually cleaved off during in vitro proteolysis.

So the important thing about this is that if your antibodies are directed against a stable region of the molecule, then you are still -- even though you may be measuring a fragment, if that fragment is on a mole-per-mole ratio, then you are

still going to have a correlation in terms of degree of damage.

Standardization. Again, in clinical size, there are a lot of troponin I assays on the market using different standards. And there is a big drive to have an international standard reference preparation.

In nonclinical studies, maybe -- because of the fact that you have a control group there -- that is less likely to be a factor.

So in terms of its validation as a biomarker -- and again, these criteria really come from my background in terms of clinical pathology. So clearly troponin I, in terms of its myocardial toxicity in nonclinical species, has demonstrated specificity, sensitivity. We do have an awareness of its half-life, particularly with acute damage. The size of the molecule is 24 kilodaltons. The study design is important because, again, if you are looking for acute damage, you may need to know when to measure it. And clearly measuring troponin I on day 7 of a study when the damage may have occurred on day one would be useless. Again, we have a feel for stability

of the molecule as well.

The methodology is good. We found it to apply to the rat, dog and the Marmoset. The mouse is the only species at the moment where we can get confounding data. Biological variation is good. Troponin I will occur in very trace amounts. You would not normally expect to see it in there.

Use of pattern recognition. Again, with the Marmoset, we can measure other cardiac markers because of the availability of assays that give cross reactivity. In the rats, we basically used histopathology to validate the appearance of the marker.

And toxicological significance, I think the marker does have direct relevance to man. If you see changes in cardiac troponin I in nonclinical species, it should certainly be measured in clinical studies.

I then move on to the protein chip technology. And again, this is a collaboration between ourselves and Glaxo Wellcome, the London School of Pharmacy and Psychogeny Biosystems. Protein chip

technology -- I know this is being talked about later on. So I will basically skip over these slides. We have protein chip arrays, protein chip reader, protein chip software. Basically, you are looking to capture proteins on the chip by two specific methods using chemical surfaces, which will be a reflection of the physicochemical properties of the proteins. So whether hydrophobic or ionic in character, or again using a biological capture mechanism, where you can place a molecule onto the surface of the chip, be it an antibody receptor or an enzyme, and look to pull out specific molecules from the sample of interest.

So basically your sample, which could be urine, a tissue homogenate -- plasma, we have been less successful with at the moment -- again, cell culture media. They can be placed onto the ship and the sample proteins combine to the chip according to using either chemical or biological docking sites.

Non-binding proteins can be washed way by a variety of buffers, and so therefore eliminating sample noise and showing some degree of on-chip purification.

Once the chip is retained, you use the process of surface enhanced laser desorption ionization to apply the laser energy to the protein that causes then the protein to fly in terms of the time of flight mass spectrometry and lower molecular weight proteins arriving that are detected first. And basically in terms of the data handling, you can capture the information, either with a spectral view or map view or by a gel view. So you have different ways of presenting the data.

In terms of when Glaxo Wellcome looked at this technology in June or July of last year, we looked at a number of different features. The one feature I will just show is where we looked at biomarker identification. The approach was to maybe hit the muscle in again Wister Han treated rats with a skeletal muscle toxicant. In this case it was 2356 tetramethylphenaminediamine. The rationale being that if you hit an organ rich in proteins, what would we see in the urine. And here is a comparison of control urines and treated urines in just this initial experiment. We can pick out this cluster of proteins

occurring between 8.5 and 12 kilodalton region.

Again, just further investigation of the technology, we looked at on-chip purification. This is the control. 50 percent acetonitrile. This is the data on the previous slide where the wash was 10 percent acetonitrile. And increasing the wash to 50 percent acetonitrile removed proteins but left us with a clear marker protein here.

So in just initial investigation of searching the protein data base, feeding in the molecular weight of 11.8 kilodaltons and other search criteria being the muscle and also rat, we pulled out this information. Water soluble calcium binding protein, rat parvalbumin thought to have evolved in relaxation skeletal muscle fibers. Again, the molecular weight very similar. But again what was also interesting is that we would use a hydrophobic chip and quite strong conditions, and we had correlations with published paper on its purification.

So we went back to further investigate this technology and did a more tighter control study. So we looked at male and female Wister Han rats given

subcutaneous injections of the TMPD at low and high dose levels. The rats were placed in their metabolism cages for 20 hours and allowed access to water only but not food. And then a number of observations were made.

This is a list of observations made. Looking at serum enzyme levels predominantly aimed at detecting muscle toxicity, looking at the quality of the urine to assure there is no renal toxicity occurring, but also a feel for the concentration or urine and urinary protein. So we applied SELDI analysis to the urine and performed histopathology on a range of tissues and also looked at SELDI analysis for the tissues.

This is the serum enzyme data, plasma enzyme data. Just some representative data between the control and high dose in the male rats. And again, this is 24 hours after dosing. So you can see clear increases in AST, aldolase. The CK is also increased and ALT is also increased. Again, in order to ensure that what we are looking at is skeletal muscle toxicity and not liver toxicity, we measured glutamate

dehydrogenase, which is again a liver specific enzyme in the rat. It is also found in high concentrations in the kidney. But if you don't have renal toxicity, most of the enzyme would flush out into the urine.

The other feature was the ratio of ALT to AST. Normally in skeletal muscle its distribution is 1 to 7 or 1 to 8. So by virtue of the ratios, the increases in aldolase and CK -- again, these CK values are not really giving you a good guideline on the degree of damage. In early work we have done with this compound, we can see increases of CK up to 50,000 or 100,000 within 12 hours of administration of the compound.

So what we basically did was create a muscle enzyme index based on the AST and aldolase elevations to give some degree of correlation of toxicity. And then we applied SELDI analysis to the urine. This is female control animals. We could see some degree -- a very small elevation of this protein appearing at 11.8 kilodaltons.

When we get to low dose in the females, we start to -- again, one or two animals show a peak.

This peak is then reproduced more clearly where we had Malamute elevation in muscle toxicity as by the muscle enzymes. And we could see a representative peak in all the treated animals. So that is the female data.

In the male data, this is a composite slide showing control, low dose and high dose. And again, showing the appearance of this peak at 11.8 kilodaltons in the urinalysis. This again reflects matched conditions generated in the early experiment using a hydrophobic chip and washing with 50 percent acetonitrile.

Just some correlation between the parvalbumin index and also the muscle enzyme index. Again, this is female data. So the parvalbumin index is a correlation of both the increase in appearance of parvalbumin related to an insulin internal standard. So what we do is send out insulin to the chip and the ratio of the parvalbumin versus insulin is calculated. But also the index related to urine concentration as well. So we took creatinine as being a factor of that.

Basically what you can see here is some appearance of parvalbumin in control animals, which

you may expect a turnover. The animals have been metabolized for 20 hours, but there has been no access to food. But we can start to see some correlation of the appearance of parvalbumin with the appearance of muscle toxicity as indicated by serum enzyme elevation.

This again -- I think you can see this. We looked at -- we performed SELDI analysis of tissue homogenates with a variety of tissues -- stomach, heart, liver, diaphragm and kidney and intercostal muscle and skeletal muscle. And we can show the appearance of the 11.8 kilodalton protein -- this is in the urine -- in the skeletal muscle and also in the intercostal muscle. We didn't actually determine the diaphragm. But you also can see that in terms of its appearance in other tissues, we weren't able to detect the protein.

However, while the data has proved very useful in terms of a proof of concept study, we are only halfway there. Yes, we have some degree of specificity of the molecule. But we really need to test it further. We are generating toxicity studies

where the skeletal muscle is not affected.

Sensitivity -- the protein appears to correlate well with evidence of muscle enzymes. Whether it is going to give any additional value is another thing. In vivo and ex-vivo half-life, again it appears within the urine within a 24-hour time period. Its continued excretion, we don't know about. The size of the molecule is 11.8 kilodaltons. But again what should be pointed out is that we have not uniquely characterized that molecule. It may be a fragment we are measuring there, but a uniquely consistent fragment. But it may also -- it may actually be the intact molecule itself. But we have not uniquely characterized it.

Study design. We are aware of the study design in terms of its appearance within a 24-hour time period, but we don't know about the design within a 7-day or a 28-day study.

Stability of the molecule. We feel it is fairly stable. But again, we have not carried out extensive stability studies. But we have revisited the samples over several months and are still able to pick

up the protein.

Robust methodology and knowledge of reproducibility. I'll come on to that in the last two slides. Biological variation, we have no information on this. Use of pattern recognition in diagnostic value. Again, we can show it does correlate with other traditional measures of muscle toxicity. But again, we would want to look at other methods of nonskeletal muscle toxicity to assess its importance.

Toxicological significance as far as relevance to man, we don't know.

In terms of the actual SELDI technology, while we were very lucky I think in some ways in actually demonstrating proof of concept with the technology, now we have got the technology in here and it is time to apply it to other applications and we recognize there is a lot of information we need to gather. This is where I think future collaborations come through.

In terms of protein expression, whether increased protein represents new synthesis or release from damaged tissue, you basically have a mixture of

proteins released. And the thing about the technology is that some of the proteins will fly and some won't. Some will ionize and some will be detected. And so, again, you don't know what you are missing. You may see what you are capturing, but you may not be aware of what you are missing. And the optimum protein concentration is going to be an important feature as is also the relative concentration of proteins in the mixture.

One thing that is important -- it may not be necessary -- it shouldn't also be diverted in picking up the protein of the major excretant in the mixture. Because it may be that the true biomarker may actually be masked by an increased protein. So there is a lot of investigation in looking at both removing proteins of large amounts and actually seeing how that preparation then looks on the chip in terms of protein peaks captured.

Purification procedures. We are looking at maybe applying pre-chip procedures versus on-chip. You may argue that this is taking away some of the beauty of the technology. But as my boss says, we want

to know what the size of the haystack is, and I think that is something that you don't necessarily know with the protein chip technology here. So apply some pre-chip purification, maybe gel filtration or isolate fractions according to a range of molecular weights and then apply it to the chip and that will maybe give us more information and strengths of limitations of the technology.

Identification. Again, we don't know if we are looking at protein fragments or actually intact protein molecules. And this is actually going to be a very important area. Because it may be that this is a functional protein, for example, which has a different molecular weight than say a rat or a dog as opposed to in the clinic, and then you will need that identification. Because it may be that that will get the transfer from the non-clinical study into the clinical study.

This is where a lot of work is actually going in now in terms of reproducing the technology where we actually are using known protein mixtures. So we are looking at quality control and chip surface,

ionization of the proteins, normalizing the data against known molecular weights proteins added to the chip, control patterns. Again, this is another area for collaboration, looking for -- building a data base of control patterns in urines and in tissue homogenates, et cetera, so you can clearly identify when something is actually going to be abnormal.

Date handling and presentation. It may be that in terms of a profile of a urine or a sample may actually be over different chip surfaces -- hydrophobic, immobilized methylphenaminediamine capture ionic. And you really want to try and pull that data together in one package and clearly display that.

Archiving of the chips. I think this is actually quite a nice feature. In terms of the chips themselves, even if they apply the laser to the chip, only part of the dry protein or the protein that is on the chip surface actually flies to the mass spec. So you have always got some protein that is retained on the chip. And it may be that if you archive the chips and go back two months later, particularly if you

tried a different procedure and identified a new protein, you could go back and see if that marker protein or marker molecule was present on some of the earlier studies.

I am sorry, I think I have probably run overtime, but thank you very much for your attention.

CHAIRMAN DOULL: Thank you.

MR. YORK: I have got some notes I can give you. I haven't got a copy of the slides with me. I have got some notes I could give you in the interim period.

DR. HOLT: Should I just go on?

CHAIRMAN DOULL: Sure. Yes, let's go right on.

DR. HOLT: Okay. I'm Gordon Holt. I am here from Oxford Glycosciences, and it is a great pleasure to use this opportunity or to be given this opportunity to come and speak to the committee today. And I want to put a special thanks in for Frank Sistare for giving us this chance. I think Michael has got my pointer, so we will see if this one works.

What I am going to do today is quickly --

and I am going to do my time thing here so I don't go too much longer -- is give you some case examples of the use of proteomics in surrogate marker identification. I apologize in advance if I am using the words inexactly for surrogate marker. I do understand that there is nomenclature that is trying to be developed now. When I say surrogate marker, hopefully the context will help you know exactly whether it is an endpoint marker or clinical evaluation or study choice or whatever.

Very briefly though, I will just touch on what the technology is. I won't give any real details about how we go about doing this. I would be happy to do that at any later date or speak to anybody individually about that. Instead, I will just kind of summarize the technology in its boldest elements right now and then give some specific bottom line details in terms of where the technology is today. What we can actually measure today.

So as the briefest of overviews of what proteomics is, in case anybody doesn't know. The concept actually hasn't changed all that much in 20

years, but the technology has advanced enormously. And I will try to give specific examples of that. But the concept is simple enough. You just take two samples, tissue or blood or whatever, and run them on 2-D gels. The first dimension is isoelectric focusing. The second dimension is SDS page, fairly standard there. Then using a variety of optic tactics, one can scan these -- these are actually scanned gels here. One can scan them into some data base and then use informatics techniques to do comparisons of the features or the spots on the gel after they have been stained. The feature comparisons then allow you to do some differential analysis. This is meant to be a histogram of looking at a given feature compared to another feature on one or two or actually quite a large group of cells it is very successfully handled.

And then depending on what the feature changes are that are seen, of course one's interest is immediately peaked about what that particular feature is. So there are tactics now that are actually quite easily industrialized to scrape those features physically off of the gel and put them into a robot

rig that will go on and make peptide fragments out of those features shown here and then pass those peptide fragments directly onto a mass spec. Once on the mass spec, there is again a variety of tactics that are well known to the art for determining the absolute sequence of those fragments, if not the peptide mass, then the absolute sequence. And then using public and private domain data bases, one can match those fragments back and forth across the data bases and end up with an absolute identification of what that feature was. So then it feeds back on itself, rounds and rounds of iterations, so that the system gets smarter and smarter over time.

Now in terms of the challenges that have faced any kind of surrogate marker identification, some of which are shown here, I am going to focus specifically on the challenges that proteomics has had to face in terms of surrogate marker identification. Sample variability is everybody's problem. It doesn't really matter who is doing what. That is, every human in the room will come in with a certain amount of variability. So that is built into that. You need to

handle a massive amount of information and make sense out of that. That is all about process validation. Of course, there is also sample handling issues that are unique to proteomics. Certainly one has to in advance know that one is going to do a proteomic sampling, because you need to stabilize the proteins. In many cases, you can't just simply put a tissue up on a plate and come back three hours later and try to do an assessment of it. That doesn't work.

Low sensitivity had been an issue historically. I think that -- while I'll give some specific examples in a moment of where sensitivity ranges are now. But again, there are a variety of tactics that we and our other proteomics colleagues have used to improve on this. Immunoaffinity enrichment, subcellular fractionation, a variety of improvements in dyes for staining the gels and also improvements in the imaging tactics themselves have brought us pretty well on scale with the kinds of things that I think are of interest to this committee.

Gel variability. Any manufactured process has built-in problems to it. We are no exception to

that. We and other companies have developed process validation tactics to make sure that we have minimized as much as possible the variabilities in putting gels between two glass plates and running them at a high voltage. In addition to that, we have also devised some imaging warping tactics to face the inevitable consequence that there always will be a few spots that will shimmy to the left or to the right a fraction of a millimeter, and we have ways of bringing these back into register so that in the end the comparison is meaningful.

Throughput has always plagued this industry. Again, it is a matter of a snapshot in time where we are today. I can tell you that my company runs a thousand gels a week. So we are at a pretty good throughput, but we are not at tens of thousands of gels a week. Although I should emphasize that the technology is clonable. So I think don't look at it today and say that is the limit. That is just today. And the goal there, of course, is robotics and people who service day and night the instruments to make sure everything is fed.

And then the data analysis overload, the same problem for us as everybody else. This is about LIMS. And without the proper LIMS, you will be absolutely buried with data. It is way past the ability to just flicker -- to just compare a couple of gels and flicker back and forth. You can't do that anymore. It simply will overwhelm you.

So some bottom line information. This is shown here in the context of genomics. Perhaps not the central focus of this committee, but it just is a point of comparison for something that people see a lot of nowadays. In terms of coverage per run, theoretically now I think everybody would concede theoretically that the time has come when genomics could get every single gene in a body, whether it be a mouse or a human, on a single chip and theoretically read all those things. Now there is still quite a bit of technology that needs to be developed, but I think that end is in sight.

In terms of proteomics, there are inherent limitations to the technology. You are at the end of the day looking at a gel. So if there are too many

spots on a gel, at least right now, that is going to confound. The gel spots or features will start to overlap. Right now operating in around 2,000 features per gel is easily handled by our technology. Now understand that is not the limit of analysis. That is just on a single gel. If you want to spread a sample out more -- for example serum -- there are a variety of -- changing your pH ranges for the separation, doing subcellular fractionation, purification and so on. So you will still get tens of thousands -- well, anyway, ten thousand features out of a single sample. You just need to spread it out a little bit more, at least using current technology.

In terms of -- I will skip sensitivity and in just a moment come back to it. In terms of protein modification, I think it is really important to emphasize that a whole aspect of our universe is in post-translation modification. Is there phosphorylation going on? Yes or no? Without that information, oftentimes you will see a change, but you won't know if it is meaningful to the cell. So certainly that is quite prominent on proteomics and

frankly can't really be assessed by genomics.

Subcellular localization, subunits in complex formation, all that are very important. Now, for the issues on sensitivity. Genomics right now is operating on one molecule of message per cell. And again, I think that is still theoretical, but that end is in sight. Proteomics today is about 100 molecules per cell. And I will give you a bottom line on what that means in terms of serum. Mostly I am going to talk about serum today. Serum analysis -- my company at least is at about 7 to 10 nanograms per ml on the scale for us. That is today. We are looking at advancing that and hopefully fairly soon will be into the range that Malcolm York just spoke about for troponin, where we can see that on a single pass gel as well.

Clinical samples I think are very important though to emphasize again. Proteomics is quite amenable to samples that are easily available in the clinic and that are realistically available in the clinic. That would include blood, serum and urine. They all work just fine on proteomics.

So I am going to go through quickly a few case studies for some work that we have done to give you an example of what the technology is useful for and what its strengths and weaknesses are. The first one I am going to talk about is the collaboration we have with Frank Sistare, who has already spoken from CDER at the FDA. For the sake of time and because this is undoubtedly a very knowledgeable committee, I won't talk too much about doxorubicin. But this was a case study that we worked on. Doxorubicin, of course, is an anti-cancer agent. It is well known to have a cardiotoxic endpoint to it though. And the cardiotoxicity seems to be mediated in part by metal ions and metal chelation by an ICRF compound. ICRF 187 seems to provide significant chemoprotection that is important in the context that we wanted to look at. So the basic question is can proteomics identify clinically relevant markers for doxorubicin toxicity? The important point with these case studies is they don't have to be elaborate at all to get good working data to move forward with. In this case, three rats per group. I think there were a total of 15 rats that

were used in this experiment. Dose is a textbook dose known to induce cardiotoxicity treated for seven weeks and then sacrificing the animals and took blood. We did do a serum purification step. To give you an example of the technology advancement, when one takes serum or plasma out of any animal, there is quite a bit of these four proteins -- albumin, haptoglobin, IGs and transferrin -- that in fact will confound the data analysis. This is an example of a 2-D gel with raw serum. Where you see these major proteins here are actually blocking up quite a bit of the gel surface, and there is a close-up view. So this is albumin and this is probably transferrin in a heavy chain. They actually totally dominate that gel. What you need to do is to get rid of them. We have developed some immunoaffinity technologies to get rid of those proteins, because they are actually not part of the disease process in almost all instances. So here is an example of a full scale gel that has been immunoaffinity purified and then the close-up. And you can see, again, that the major point is that not only can you physically load more protein on the gel and

get to see a lot of the other things that might have been missed, but you can also remove these clouding elements -- these occlusions that would otherwise eclipse the proteins of interest. These proteins are not albumin and IG and so on.

So what kind of assay we did on the samples? We ran one gel per plasma sample. This PEM is protein expression map or gels in our hands. We looked at approximately 1,800 features in every gel at all times across the board. In this particular quite a small study, we looked at about 32,000 features, screening compared to each other. We set a rather high stringency for the comparison. In this case, we chose a 98 percent marker confidence. That is that the markers had to be well on scale and we could easily discern what changes were occurring. And the 100 percent incidence, that is, every single animal in every single group had to either have that feature there or not there or changing in synchrony. So in this case, these markers would be quite robust.

As markers, what did we find? On doxorubicin-induced toxicity versus control, we found

about 34 markers that deflected the midline here as abnormal. Remember again that each of these proteins has their own baseline for what is normal for that protein. So this is all just expressed as a fold change. So we found about 34 markers that changed compared to normal in this study. When we used ICRF -- you remember I spoke about ICRF as a chemoprotectant against doxorubicin-induced toxicity. We are very pleased to see that almost all the markers went back to their normal values. A few of them did not. Looking at it together, again you see all these basically went back to normal, a few did not.

So what is the bottom line on this? We found 34 markers that seemed to be consistent with doxorubicin-induced toxicity, some of them quite profound changes. It appears that these are excellent places to start for clinical markers of doxorubicin-induced cardiotoxicity. And another important point, the technology itself also demonstrated using the ICRF as an internal control that ICRF -- not only does it show that ICRF looks like it is a pretty good chemoprotective agent, but I think this is an

important point too. What are the markers that actually change? We have gone on and sequenced them. Without going into details right now, I would just say that they followed along the lines of lipid metabolism, in particular liposome formation.

Immunosurveillance. There was quite a few markers that were involved in complement fixation. Wound healing. Of course not surprisingly, we saw some proteins involved in scar formation. Proteolysis of protein components in the cells. And then this is a very interesting finding too. We found some anti-oxidant proteins being deflected from normal, in particular some metal scavenging enzymes. And this, frankly, is a bit of a surprise to us. What it showed is doxorubicin does depress in this case the metal scavenging proteins, and I guess that is known in the literature. But interestingly, it looks like that sort of demonstrates the motive toxicity.

I will speak very briefly about other projects we are doing with Frank Sistare. We are also looking at vasculitis markers in a case study. In this case, we are looking at SKF, a compound that is well

known to induce vasculitis. We are doing a time course study. We will -- actually, these gels have already been run and hopefully very soon we will be able to give you some data on that if you are interested.

So that is about serum markers for cardiotoxicity and vasculitis. That is not the only thing we are doing. We are also looking at marker -- looking for surrogate markers with in this case the CRO quintiles. Looking in particular for surrogate markers that are consistent with nephrotoxicity. Again, I don't need to tell this group that much about gentamicin. But gentamicin is our case study in this instance, where it is a good antibiotic and it also is well known to have ototoxicity endpoints and a reversible although sometimes irreversible kidney toxicity.

This gives an example of the more robust study design that we and others would like to do. We have actually done this study now where we are looking at quite a few dose levels and large group numbers. We are looking at standard clinical values for toxicity. And we are running quite a few samples, in this case

420 samples, looking for changes. I didn't bring data to present on what is found other than to just -- what is found in the non-proteomic arena. I will just simply summarize it by saying we found what the textbook would suggest. That you see nephrotoxicity at about 40 to 60 mgs per kg. We see that histologically, and we also see that in the expression of some of these classic clinical markers of nephrotoxicity.

What did proteomics show? I will just show you a very scant bit of data on just the serum analysis. In this case, we looked at 30 different images or 30 different samples. 2,500 features were followed simultaneously. We looked at about 21,000 features for consistencies and change.

What is the bottom line? The bottom line in this case is very unusual, I should emphasize. We found a single protein that seemed to correlate across the board with the emergence of nephrotoxicity. This particular protein is involved in the alternate pathway of complement. Is it totally unanticipated by the literature? Not exactly. Although in all

fairness, it is known that the alternate complement pathway is involved in this procedure. But I think it is fair to say that the literature certainly didn't anticipate that a single protein would be such a strong predictor of kidney tox induced by gentamicin in this case. A very critical finding. What we found is that once we knew what the protein was and we started looking at lower and lower doses, we could actually see that this particular complement protein deflected from normal at a dose even lower than histopathology, the other standard clinical markers of kidney toxicity emerged. That is a key finding. Obviously what you want to do is to catch something quite early, well before you actually have serious damage.

So the technology is not only useful for looking at toxicity. It also, I think, is quite sturdy at identifying patients who should be treated with something. And this is just an example of one of many projects we are conducting at OGS to look at actual human samples in this case to try to distinguish different patient groups at points of critical

importance in clinic -- should I treat or should I not treat or should I go to a more strenuous test or not? So this study was done with Dr. Coombs at the CRC looking at breast cancer sera. Again, a key point is you don't have to do a very large study to get a very compelling data set to at least begin a very large study with.

In this case, we looked at sera from 17 normal patients, 17 patients with diagnosed primary breast tumors, and then 17 patients with metastatic cancer. And we did apply the serum enrichment protocol that I talked about a moment ago on these samples. What did we find? Again, this is all focused on bottom line. What we set for our criteria for acceptance in this study was a P value that was approaching fairly significant statistical significance, a P value of less than .005 for that feature, and an incidence of greater than 50 percent. So at least half of the patients had to show this marker change compared to the patient group to which that particular feature was being compared.

We identified 63 potential surrogate

marker proteins in this study. In terms of what the metrics are or what they are. In the normal versus primary metastatic cancer, we found 16 proteins in a differential, normal versus metastatic 20. And I think this is probably the most important set of this group personally. And that is primary cancer versus metastatic, we found 27 different proteins. Now what does this mean? Actually, it is a very important clinical decision making of whether to decide to treat someone with rather standard technologies with standard treatments for primary cancer, or whether it is time to go right into the very heavy hitter and in fact much more risky treatments for metastatic cancer. And again, this is a clinical decision making point, and it looks like we have a pretty good set of markers to look at here.

CHAIRMAN DOULL: Did those overlap?

DR. HOLT: Most of them do not overlap interestingly. The question was do the proteins overlap, and most of them don't.

Just a brief summary. I will just again go to the bottom line. Hopefully, I have persuaded you to

at least consider proteomics as a powerful tool for surrogate marker identification. Frank asked me to speak to this, and I agree. I think it is very important to mention that we have had quite a lot of information from various sources about the need for having some kind of consortium to work together. I think I can say that certainly my company and I am sure most would seriously consider joining into the consortium. I wanted to flag the critical issues in case it is not entirely obvious. But there is no question that given the institutional mandates of the major players, they are all -- FDA needs to be doing its own charge and the academic and industrial partners all have their own charges. I think it is inevitable that all these players, if they are to come together, are going to have to work to the same endpoint for different motivations. Because I don't think there is ever going to be a way to overlap everything. I think it is also inevitable that there are going to be stage specific pressures. The pressures for discovery are different than validation and commercialization and so on. And I think these

are going to have to be addressed even before it is started. And last but not least, this is not a stopping point. It is simply a point of acknowledgement that intellectual property ownership is going to have to be settled very early and very quickly. And I think one thing that is going to be key is that the discoverers in most instances are probably not going to be the developers of the information.

With that I will stop and pass. Any questions?

CHAIRMAN DOULL: Thank you, Dr. Holt.

DR. GOODMAN: Can I ask a question, John? Could I ask a question?

DR. HOLT: Yes.

DR. GOODMAN: For the doxorubicin and gentamicin?

DR. HOLT: Yes.

DR. GOODMAN: With the dosing schedules that you employed?

DR. HOLT: Yes.

DR. GOODMAN: How did proteomics tell us anything different than using measurements -- standard

measurements for serum transaminase and/or standard measurements for urinary electrolytes or gross protein in the urine?

DR. HOLT: I mean, speaking directly to serum transaminase, none of those proteins were serum transaminase. I have no idea --

DR. GOODMAN: No, I understand that. But if we did the standard, routine, basic clinical serum analysis or urinalysis, how would we have learned -- would we have learned -- how did proteomics tell us more than that standard basic analysis?

DR. HOLT: That is fine. So in terms of the quintile study I think I showed -- and again, I would be happy to put some hard data up if you want to at some future point -- but we certainly found that we could identify markers emerging at a concentration that no other parameter showed a change compared to normal. That includes all the items that you just listed. So we were able to see a deflection even before that. Why is that? It is probably because there is a metabolic change within the cells, even before they were burst and even before there was major tissue

damage that you could see the changes.

DR. GOODMAN: But I don't understand. When you say before, do you mean before in terms of time or at lower doses?

DR. HOLT: At an earlier concentration. I am sorry, it is imprecise language. At a lower concentration than you could see damage otherwise. In terms of time, I don't remember off the top of my head. But it wouldn't surprise me at all if you saw it.

DR. GOODMAN: But maybe the lower concentration doesn't really produce toxicity, so you could get a false positive.

DR. HOLT: That is a great point. I mean, I think efficacy and toxicity are always one of these things that go back and forth. But it certainly -- those markers clearly are related to toxicity at higher concentrations. Are they related to toxicity at lower concentrations? I think that that is what one needs to study in detail.

I wanted to point out too on the doxorubicin study, again in fairness, I think that

many of the proteins that we saw changed actually not only speak to the toxicity, but in the case of doxorubicin appear at least at first blush to speak to the mechanism of toxicity. Again, I don't think that you get that kind of information from the standard techniques that are used now.

DR. MacGREGOR: I think I could add to that question actually. In the case of doxorubicin, troponin T has in fact come into clinical use because clinically it is the most reliable marker for doxorubicin toxicity, and it is used to gauge pediatric chemotherapy for that reason. So I think it is actually a good example of how these technologies can turn up new candidates that you can then assess one against the other and optimize the best uses.

DR. GOODMAN: Well, I understand. But the real direct, direct issue is -- and I don't mean to be facetious -- but not to find a more expensive way to do what we currently do routinely.

CHAIRMAN DOULL: Yes, but it might be predictive, Jay. You know, the test might be more predictive than the transaminase. It would predict it

earlier. Yes, Joy?

DR. CAVAGNARO: For the doxorubicin then, the histogram or cartoon that was presented, did you note any one of those as proponents?

DR. HOLT: None of them. In fact, I will speak directly to that. I don't think troponin will be on our gels. I think that from what I understand of the concentration, that it is supposed to deflect. I would have been surprised had it been on the gels that we saw. We are approximately five to ten fold less sensitive than to be able to pick up troponin in a first pass. I want to emphasize that that is a first pass. If you want to see troponin, then what we would probably do is to decrease the -- use another immunoaffinity tactic to get rid of other proteins. We would probably change the pH ranges so that we would see it directly on the gels.

CHAIRMAN DOULL: We will go ahead and continue the proteomics discussion with Dr. Anderson.

DR. ANDERSON: Thank you. I'm going to attempt to use this very good sound system to be heard to you today despite laryngitis, and I will also try

to be as brief as possible but convey to you basically three things. First, I would like to discuss a little bit the basis for the belief that protein studies are a profoundly useful path to discover markers. Talk a little bit about the technology and then show a series of case studies in which the technology has been used to find novel markers of toxicity, particularly in rodents.

Now the issue with genomics transcriptomics as it is now called and proteomics is really that the genome is telling us what could possibly happen. It is the plans for an organism. Messenger RNA measurements tell us what might be happening, because messages only have the function of specifying the production of proteins. And proteins actually are the elements of biological systems that function. And protein abundances therefore tell us what is happening in a biological system.

Now what we really want to understand obviously is functional change, both the pharmacological and toxicological aspects of functional change. And in percolating information

through from the genome to message to protein to function, clearly we have some questions about the translation of those potential markers here which need to be resolved at the protein layer. And not only that, we have changes in the structure of proteins which in fact can't be observed at these other layers.

Now I want to make the point that it is fairly important to realize that protein and messenger RNA abundances are not very well correlated. And, therefore, if one has to choose a molecule to measure as the diagnostic marker, it is much more important to measure the one that is proximate to function. And I would point out that several studies, the first one of which we did in collaboration with Incyte Pharmaceuticals, to look at the correlation between message and protein give very poor correlations. The second major point that was emphasized earlier is that important sample types just don't contain useful RNA.

The data in comparing message and protein abundance gives an obvious impression of lack of strong correlation. This is the abundance of a protein versus the abundance of its specific message

in human liver in this case for a series of different gene products. The correlation is a little bit less than .5, and there is a tremendous amount of scatter, which is in fact not method-dependent. It is found in a whole series of studies which indicate that this correlation is poor. Therefore, protein measurements are going to be very important to us. And what we would like to be able to do is to measure the protein fingerprint and see how it is affected by things like perturbations around the normal state. These can comprise disease states or treatment effects. And we can divide these. This is to some extent a philosophical distinction into therapeutic and toxic effects. And we can look at progressive change in the normal state, which is a more academic enterprise looking at differentiation, evolution, et cetera. But for the current purposes, we are interested in the effects of drugs.

Now drug effects are expected to have representations and changes in the amount of messenger RNA and proteins for many proteins because of the fact that a lot of regulation exists in biological systems.

If you inhibit an enzyme, things happen. You get regulatory changes that cause changes here. The same with receptor binding or blockade of a channel.

Now in order to measure effects like this, the technology that is required really comprises four components, and I will very briefly mention what these are. Experiment design, sample fractionation for example, is extremely important, as Gordon has previously mentioned. 2-D gels are still the core technology here, although it is very important to realize that over the longer term we are talking about 2-D gels and proteomics as a discovery technology which will generate markers which percolate through to all the normal measurement methodologies we want ultimately leading to something that may be termed protein chips, the ability to measure large numbers of proteins specifically and cheaply. Mass spectrometry is used for identification of the proteins, and software is a major component because of the amount of data that is involved.

I will just make one very important point about 2-D gels. These are separations of many

proteins. Each spot is a protein separated by isoelectric point and molecular weight. And the abundance of each of these, the integrated absorbance of it in this particular case, is a representation of the abundance of that protein and hence is equivalent to a specific test for a specific protein molecule. So we are running specific tests for large numbers of proteins in a discovery mode.

2-D gel resolution has a profound effect on the data quality, and that is the reason why it has taken a very high degree of automation to allow us to go from situations like this running gels essentially by hand to being able to distinguish all the protein spots and measure them independently by automated methods. And even the shapes of these little spots is very significant. In the very high throughput systems, these are Gaussian spot shapes, as they should be, determined by diffusion of the proteins.

Mass spectrometry is basically fed by systems in which gels are taken and the proteins are excised by mechanical systems and put in 96 well plates for high throughput processing and analyzed by

mass spectrometry. And ultimately, the data flows into a data base. In our design, we use two data bases. The first is human, looking effectively at disease processes, molecular anatomy and pathology data base. In parallel with that, we look at drug effects, primarily in rodents in the molecular effects of drugs data base. And of course the bottom line is the relationship between these two, because we want to develop drugs which reverse disease processes.

Now so far we have looked at a little over 50 pharmaceuticals which are in the PDR in rodents to look at the effects in certain tissues in order to develop a background data base. Altogether in the commercial as well as in our own projects, we have looked at about 100 compounds so far. This is a representation which I don't expect you to be able to read of a summary of the results of changes in this subset of proteins caused by the effects of 182 different drug treatment groups here. This is about a quarter of a million different protein measurements compacted together and analyzed in such a way that we are clustering them. I will show you what is in the

little box. This is a set of proteins which are affected by a set of particular drug treatments. And these blocks of color in representations like this allow us to begin to see the relationships between drugs and the relationships between proteins.

Now let me very briefly illustrate for you a series of case studies of the application of this technology to the determination of -- the discovery of protein markers and looking at mechanisms associated with specific effects, including mainly toxicities. I will show you the comparison of therapeutic and toxic mechanisms within a class of drugs, cholesterol lowering agents. I will look at an SAR study, the PPAR alpha nuclear receptor compounds, the peroxisome proliferators. Recognizing the mechanism that you expect in one class of compounds to appear within a different class. Looking at the relationship between toxic and therapeutic mechanisms in the well-known case of cyclosporin toxicity. And then looking at covalent protein adducts and the different series of covalent adducts, which is something that is peculiarly possible with proteomic technology.

In the case of cholesterol metabolism, there are a series of good drugs which regulate cholesterol. The statins are the primary class used in this application, but it can also be regulated by cholestryramine by a different mechanism. And we can achieve the opposite effect by feeding a high cholesterol diet, in this case to F-344 rats. I would like to point out that this is a montage of many of these two dimensional separations of liver proteins from rodents. A small proportion of the same pattern for each individual animal. And the yellow arrows show a particular protein spot which is progressively increased from no abundance in a high cholesterol diet through the controls and progressively increasing to the synergistic combination therapy with these two compounds. When this protein is identified, it turns out to be HMG-CoA synthase, the enzyme immediately prior in the cholesterol synthesis pathway to the target of this drug. That is not surprising. We should be inducing elements of that pathway. And that in fact is a confirmation of the fact that we are seeing effects which are mechanistically valid. The

little green circles here are circle spots which are affected strongly by this sequence of treatments. These are the same five experimental groups as in the previous slide. So this is a combination therapy high cholesterol for a series of different proteins which are obviously coordinately regulated. They show the same response profile across the animals.

Now there is in fact a different response in the little blue box here, which is a protein which is induced more strongly by lovastatin than by the same dose of lovastatin plus cholestyramine, an anti-synergistic regulatory effect. That protein turns out to be the most sensitive indicator of peroxisome proliferation in the liver. So this is a measurement of toxicity. These are measurements of therapeutic effect. And we can look for a series of compounds at the ratio between the induction of the therapeutic effect versus the induction of the toxic effect and effectively generate therapeutic indices based on these proteins as markers of the performance of individual pathways. This is indicated in this study of peroxisome proliferators which we did with Eli

Lilly. Peroxisome proliferators bind to PPAR alpha. They occur in numerous disparate structural classes and are problematical. They produce liver tumors but apparently have no in vivo genotoxicity that has been determined.

Now in this study with Eli Lilly, we did 42 treatment groups, about 300 analyses, about a quarter of a million protein measurements again in this case. And we determined that more than 100 proteins showed very significant changes. P .001 in our experience is really the effective probability for a very high confidence marker at this level because we are looking at so many different proteins. When you make the appropriate statistical corrections, you come up with this kind of number.

These were the compounds that we looked at, which included five strong peroxisome proliferators and one compound that is not a strong proliferator but has the same pharmacology as this compound, its analog, which is a strong proliferator. So this was a negative control.

I will show you a kind of plot which

allows us to summarize the effects of these drugs on the abundance of many proteins. This is a 2-D gel of mouse liver, lots of proteins. The arrows emerge from spots that showed quantitative changes. The arrow is tilted upwards. The protein was increased in amount. This tilted downwards is decreased. The arrow length is proportional to the P value against controls. So this is a complex pattern of effects which involves up and down regulation of many proteins. But it allows us to look by comparing different colors at two different compounds. And obviously Nafenopin and WY14643, both prototypical peroxisome proliferators cause very similar effects. Arrows are either down-regulated or up-regulated in parallel with all of these markers. We can look at a whole series of compounds, and it is evident that all of the compounds cause very similar effects except the little magenta arrows, which in many cases are discordant. And that is because that represents the negative control compound which acts by a different mechanism. In fact, this can be looked at by classical multivariate statistics, and the entire pattern of shifts in

abundance that I showed in the previous slide boils down to one access or one measurement on each animal's gene expression pattern or protein expression pattern, which separates the controls from progressively more potent peroxisome proliferators on this axis. And a separate completely different axis of change representing a completely different pattern of protein changes separates the control animals liver protein patterns from the patterns of the animals treated with the negative control compound have a different mechanism. And in this case, it is not plotted here but there is in fact a third mechanism that can be differentiated in the experiment, which is the age of the animals. Because we did both 5 and 35-day studies.

It is possible not only to recognize a mechanism of action by its effect on multiple markers, but to recognize a mechanism where you don't expect to see it. In this study, which was done with the Cancer Chemo Prevention Branch at NCI, we looked at a series of compounds which are in clinical trials, at least one of which is in clinical trials in China, for the

prevention of aflatoxin-induced liver cancer as a series of analogues. And when we look at the effects of these compounds in a similar kind of plot, where this is an index of change over many proteins which together are Phase II enzyme inducer markers, the controls are here and progressively more potent. Phase II enzyme inducers have displaced in this direction. But one of the analogues is displaced -- the R pattern here is displaced according to a different mechanism, which initially we had no idea about. But looking at a larger data base and a larger number of controls, we have a second set of animals whose protein patterns are displaced in the same direction, and those turn out to have been treated with piroxicam. So in fact we have seen similar effects to those produced in the liver by piroxicam with a member of the class of dithiolethiones, in which that would not have been anticipated.

Cyclosporin, obviously, is an extremely important immunosuppressive drug used to prevent organ graft rejection and in collaboration with a group at Novartis in Basel, we looked at the effects of