HEREDITARY DYSAUTONOMIAS
CURRENT KNOWLEDGE AND COLLABORATIONS FOR THE FUTURE

NATIONAL INSTITUTE OF NEUROLOGICAL DISORDERS AND STROKE
OFFICE OF RARE DISEASES
NATIONAL INSTITUTES OF HEALTH

8:40 a.m.
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Plaza I
Doubletree Hotel
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C O N T E N T S (Continued)

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P R O C E E D I N G S

(8:40 a.m.)

DR. GWINN-HARDY: Hello and welcome, ladies and gentlemen to the Hereditary Dysautonomias Workshop. If I can't say it, hopefully at least I'll be able to learn something today from all of you. And welcome to all the scientists and all the other interested people who are attending today.

This workshop is sponsored by the National Institute of Neurological Disorders and Stroke and the Office of Rare Diseases. We also have with us Dr. Guttmacher from the Genome Institute.

I'd first like to introduce Dr. Audrey Penn, our acting Director at the NINDS, who is first and foremost a

neurologist and always a scientist. Thank you, Audrey.

DR. PENN: Good morning. Thank you, Katrina. I thank all of you for coming to this. I should give really very specific and special thanks to the organizers of this. We did talk about the organization and the program a great deal, and I think it is going to be a magnificent meeting. I will say -- and Steve has the same problem -- I won't be able to stay for all of it, but I know everyone here will benefit from it and be able to have a lot of interchange.

So I was introduced to this disorder when I was

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a medical student because Dr. Riley was on the staff of pediatrics at Babies' Hospital. And it was a prize disorder. Even though it's miserable, it was a prize disorder for teaching medical students, and we heard rather a lot about it. And then I have to say I didn't hear a lot about it, and it is very rare, which is why Steve is here.

But, on the other hand, for us at NINDS right now, it's an excellent example of our over 250 small genetic, mendelian genetic disorders of the nervous system, both peripheral and central, and Lord knows, in muscle too. So it's very important as an example of where we are, where we have to go.

We are very concerned with these disorders. Right now we plan all kinds of programs and initiatives for various stages along the line; that is, find the gene locus, then the gene, the coded protein, and then get the models going, and then see where we can help develop therapies. So we have a brand new or relatively brand new, big program in the institute of translational research programs, and you will see in the NIH guide the set of specific applications for cooperative agreements to achieve all of this. Clearly different disorders are in different stages in this process, but I know now that you have not only the longest, but you have a gene. Pretty

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soon you'll have a protein. So we might as well see what the models look like. So we are investing in this.

And what I am trying to say is that your issue is an issue for, again, many other disorders that we have to fix. And there are all kinds of things about gene therapy that you're well aware of that are going to make this difficult and a challenge. We do work with the FDA on these issues. We will work with the RAC on these issues, but we have to put those genes back and we have to figure out how to do it safely. And we are well aware of that too. We have had setbacks, but we are very determined to get this sort of thing done.

I am not entirely sure whether stem cells are pertinent here or not, but it's another one that we spend a lot of time thinking about, particularly because, for all of us, we're talking about the brain, the central nervous system and how to get things back safely.

So I'm really glad you all came together to look at this disorder, and I think you know a lot. And I think

you're all together, which is just great. We have some disorders where our lay community is not exactly all on the same page, but I think this one is. So I'm trusting that from this will come not only a rechallenge to us, but real progress in this disorder. You know, whatever it is. 60 years since Dr. Riley. Let's get on with it.

Thank you. Have a good day.

(Applause.)

DR. GWINN-HARDY: Thank you, Dr. Penn.

Now allow me to introduce Dr. Groft, the Director of the Office of Rare Diseases. Dr. Groft.

DR. GROFT: Thank you very much, Katrina. And I would like to thank, first, the organizers, Katrina, but also the various chairs of the sessions: Dr. Axelrod, Dr. Goldstein, and Dr. Robertson. It is quite an effort to pull one of these meetings together, and a lot of time and thought goes into the preparation of the speakers and the agenda. So thank you very much for your contributions. These are not easy conferences to pull off. It is a lot of work.

I'd also like to thank all of the attendees who have agreed to come in and share their expertise and experience with each other.

The Office of Rare Diseases is not a large office. In fact, we're very small. Some of your grants probably exceeded our annual budget of $2 million until last year when, all of a sudden, we got this magnificent jump up to $10 million. What a fortune for us.

We've implemented a number of programs. One is our intramural research program that we started, and I'd just like to introduce Dr. Bill Gahl, who's in the back of

the room, who is also the Clinical Director for the Human Genome Research Institute. He's our intramural research program director.

We will be implementing an extramural program within the coming years to fund centers of emphasis. There's some legislation moving forth in Congress to provide some additional funds to the office, and one of the major areas will be funding these centers of emphasis or excellence throughout the country and groups of rare diseases. So we're looking forward to that. Next year we hope to support maybe seven, and if our budget were to increase, we look forward to funding about 15 of these within about two years. So if everything holds, as we're planning, it should be an exciting time for us.

One of the few things, with such a limited budget, that we've been able to do over the years is support workshops such as this. We contribute a small amount of money. It doesn't nearly cover the cost, usually in the range of $20,000 to $25,000, just enough to bring in several speakers and provide some of the facilities and what have you.

But what comes out of these workshops really depends on what you contribute and what you bring into the meeting, how much you want to work together, how much you

want to share. We encourage the organizers of the meeting to get as many different experts, as many different specialties to come here and talk about their research and to try to tie together the various aspects. With so many of the rare diseases, there are so many organs or systems that are involved, that we need different approaches, different ideas, and I think we can learn from each other. And that's what we try to do in the workshops, is bring a lot of different people together to talk about what is needed. This, in essence, becomes the research agenda for the institute.

I know the neurology institute has made an unbelievable commitment over the years to these rare diseases. In fact, at one point we called it the National Institute on Rare Diseases, and we set up our office. We said we're going to take the rare diseases. They do so much, and they probably are the biggest user of our workshops program.

And what comes out of this then hopefully are grant applications from you, the research community, into the institute that they can consider. I think that's one of the goals that we'd like to see today is an increase of grants coming from you, an increase of ideas and direction to the institute and to the office. We'll see where we can go in the future, as far as all of the funding.

So I don't want to delay the onset of the meeting. I'd like to thank all of you for coming. Traveling is not an easy task these days for everyone. So it really is appreciated that you're able to come in and spend some time with us here and give us the benefit of all your experience.

So thank you, and in particular, thanks to Katrina. She did a tremendous job pulling this whole thing together in some difficult circumstances.

(Applause.)

DR. GWINN-HARDY: Thanks, Dr. Groft. Although I'm not sure how I feel about having the National Institute of Neurological Disorders and Stroke called the National Institute of Rare Diseases, because that says "NIRD" which doesn't sound very good.

(Laughter.)

DR. GWINN-HARDY: But I guess we can handle it if it means we do a lot of good science.

I want to also introduce Math Cuajungco. Is that the right way to say your name? Dr. Cuajungco is standing. He's the person who is actually writing the meeting summary for us. So I'd like all the speakers and moderators of the discussions to share their slides, as well as their notes, with him so that he can put together the most effective summary of the meeting for us.

I'd also like to ask each of the moderators to make sure they get one or two points from each of the lectures so that during the discussion and especially during the final discussion, these points can be made and

shared with others, so we have a nice summary at the end of the meeting of our goals and directions for the future as well.

Without further ado, I'd like to introduce Dr. Felicia Axelrod, our chair of the first session. Dr. Axelrod, thank you very much.

(Applause.)

DR. AXELROD: I also would like just start off thanking a few people.

First of all, I'd like to thank Dr. Sonia Peltzer and the FD Hope group for initiating contact with the NIH and providing the impetus for this important conference.

And I also would like to thank Dr. Katrina Gwinn-Hardy for her superlative efforts in coordinating and actually bringing this conference to fruition.

And finally, I'd like to express my appreciation to the NIH and really for being sensitive to the importance of this subject matter and hosting this symposium.

Really, until recently, interest and financial support for autonomic research really has been less than

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enthusiastic because it was perceived that the autonomic disorders affected only a select few, and support primarily came from small, but critical organizations like the Dysautonomia Foundation. And for almost 50 years, the Dysautonomia Foundation has really been the sole resource for financial as well as motivational support to promote clinical and basic research for FD. But now that we're here at an NIH-sponsored forum, I think that this indicates that interest in the autonomic nervous system is growing.

The hereditary dysautonomias provide a unique opportunity for scientists to obtain greater insight into the complexities, as well as the pervasive nature of the autonomic nervous system. And each of the hereditary dysautonomias really affect autonomic dysfunction, but the time of onset, as well as the extent of impairment, and subsequent manifestations really depend upon the specific genetic abnormality.

Over the next day-and-a-half, hopefully each of us will widen our particular focus by learning about the different entities, their genotypes, their phenotypes so that we will be stimulated to try new approaches, establish new collaborations. And the result of our discussion should be really a cross-fertilization of scientific knowledge so that the patients and their

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families who suffer with these disorders on a daily basis will feel that our efforts have been positive and have the desired long-term effects.

The first of these three sessions will, of course, be devoted to familial dysautonomia, and I think that this really is an appropriate place to start because this is the first of the hereditary dysautonomias to be well described and extensively studied. It affects a

genetically and pathologically homogeneous population and therefore can be considered an excellent model to understand the mechanisms involved in development and survival of the autonomic nervous system.

I will start off by providing an overview on the history, the pathology, as well as describing some of the clinical features of the disorder and how we maintain patient data so that we can evaluate clinical course and treatment and how this will be eventually the way we could possibly approach treatment therapy.

The other speakers this morning will provide further information regarding current knowledge of the disorder, as well as provide some thoughts on possible future directions for FD research.

Well, it all began about 53 years ago when Drs. Riley and Day reported five cases of central autonomic dysfunction with defective lacrimation, and you can see

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that he really stressed "central" autonomic dysfunction at this time. Since that time, we realized that there also are sensory perturbations. He did not really describe in that paper any problems with pain and temperature perception, which these patients do have.

We also know that the autonomic problems extend more than he described at that time. They involve oropharyngeal incoordination, poor speech, esophageal dysmotility, insensitivity to hypoxia and hypercapnia, as well as blood pressure lability.

The other central problems really involve peripheral as well as central manifestations of autonomic dysfunction. There are eye problems, as well as general hypotonia, and gait problems.

Interestingly, intellect is usually normal, but there are particular characteristic features. Visual skills tend to exceed verbal skills, and there are problems with executive planning and organizational skills which tend to be frontal lobe problems.

The clinical features are present from birth. Multiple systems are affected secondarily, and the expression varies widely. These are two different individuals, both 16 years of age, and you can see this young man really barely has any physical abnormalities that might be discernible to a pediatrician, whereas this

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young man is very short in stature, has severe spinal curvature. You can't see it on this slide, but he's drooling and has mottling of the skin.

Now, until the last two years, diagnosis was really based on clinical signs and there had to be evidence of sensory as well as autonomic dysfunction, and we used five diagnostic criteria. The first three were all described by Riley in his original report: the lack of overflow tears or absent lacrimation with emotional crying, the fact that there was decreased deep tendon reflexes, and as he noted, all five of his children were Ashkenazi Jewish extraction from the Eastern European area which the Ashkenazi Jews are from.

Then in the 1960s, Drs. Smith and Dancis described the tongue findings, and that became the fourth of the diagnostic criteria. In a normal individual, there are highly vascularized papillae on the tip of the tongue. This is lacking in the FD patient, giving the tip of the tongue a smooth, glistening appearance.

The fifth sign that was also described in the early 1960's by Drs. Dancis and Smith was the abnormal axon flare with intradermal histamine. And in the FD patient, the intradermal injection of histamine gives you really a very circumscribed area of redness where in the normal individual, there's a diffuse area of erythema,

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which is the axon flare.

Interestingly, this particular sign is present in all of the hereditary sensory and autonomic neuropathies, which is the other types V, IV, I, and II. Dysautonomia is type III of these hereditary sensory and autonomic neuropathies.

So in questionable cases, for instance, if we were sent a patient who seemed to have most of these signs and had an abnormal histamine test and if their parents weren't Jewish, well, then we would suggest doing a sural nerve biopsy, and based on pathological findings in the sural nerve biopsy, we were able to differentiate the disorder. However, as of 1993, with haplotype analysis and now in 2001, DNA diagnosis is possible, and definitive diagnoses can be given in this way.

So as of 2001, we had the FD gene identification confirmed. IKBKAP is the gene. IKAP is the protein product. We know that two mutations can cause FD, and we have tissue-specific expression.

So what now? Well, we have carrier testing in the general population, and this has had a tremendous impact for the patients, their families, and for the general population at large.

But there is a bigger job now, and that's to start the search for gene function. What is the role of

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IKAP and its associated protein in the development and the maintenance of the sensory and the autonomic nervous system? And how can we correlate the previous information with these new genetic findings? What about the pathological reports that we had until this time and the observed clinical symptoms? Of course, the final goal will be to develop definitive therapies.

Well, in 1958 to 1978, really before we had pathological findings reported, most of the efforts were spent on looking at catecholamines, and it appeared that the products of dopamine were elevated and the end product of norepinephrine was decreased in the affected individual, and when you gave these particular drugs, there were abnormal physiologic responses. So based on that without any pathological findings, they said patients were acting like they had denervated hypersensitivity and that there was probably a problem in norepinephrine synthesis, release, or degradation. They assumed that

this was secondary to a decreased peripheral sympathetic system.

And then in 1971, we had the first report of pathological findings, and that was really by Dr. Aguayo in Canada, and he showed that the unmyelinated and small myelinated neuronal population was decreased in the patient with FD, and this was in a sural nerve.

Dr. John Pearson at NYU then looked at sympathetic ganglia and he found the same thing, the decreased numbers of neurons in cervical sympathetic ganglia, and when he actually did counts, he saw that the FD population has about a tenth of the normal neuronal population in their sensory as well as their cervical ganglia and minimal involvement of the parasympathetic ganglia, at least the ones that he looked at.

Since that time, there has been more investigation, and this has been confirmed, again the sympathetic chain has been looked at, the dorsal root ganglia, and we have also found the same findings of decreased innervation in vascular nerve terminals which probably accounts for the vasomotor instability in these patients. But really, where no one has been able to look or give us accurate information is the brain.

So the consistent pathological findings show inadequate development of small myelinated and unmyelinated neurons in the sensory system, the autonomic nervous system, and one cranial nerve that has been looked at is the sphenopalatine ganglia. We also know it's a progressive disorder because there are residual nodules in dorsal root ganglia.

So this seems to be a neurodevelopmental and a progressive disorder. There is consistent peripheral neuropathology, but we've never been able to document central neuropathology.

Yet, we know that there are autonomic tracks in ganglia, as highlighted in red here on this cartoon, and we wonder now whether the peripheral nervous system is affected primarily or secondarily, and how can we account for the wide variability of clinical expression? Is it just differences in neuronal number, differences in neurotransmitter? And which of the genes and peptides might be secondarily affected?

In treatment challenges, there are really four that I struggle with and Dr. Maayan struggles with on a daily basis. The gastrointestinal system. The GI dysfunction in FD is prominent and probably one of the symptoms that plagues the families the most. The oropharyngeal incoordination is seen at birth and the children have difficulty feeding. There's esophageal dysmotility, as well as gastric dysfunction, but it's the vomiting or the protracted retching which significantly impacts on the quality of the patient's life.

Crisis can occur in any FD patient with sufficient stress. 40 percent will have a cyclical pattern that usually starts in the morning after arousal,

and the pattern tends to be consistent for an individual. Either it occurs daily, weekly, or even monthly. The

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stressors can be physiologic, they can be emotional, which suggests again central problems, and again, this constant theme that it occurs with arousal from sleep.

But what is a dysautonomic crisis? Well, it's a constellation of symptoms. Yes, there are GI manifestations. They don't want to swallow. They drool during these crises. They develop an ileus. Nausea and retching are prominent. The vomiting is only the tip of the iceberg. The nausea is really what they can't stand more than the vomiting. There are also cardiovascular perturbations, as well as increased secretions. The whole child is wet, and the personality changes.

When we looked at serum catecholamines during an FD crisis, we were struck by the fact that -- these are three determinations early in crisis -- the dopamine levels were extremely high. With that information, we went to Dr. Pearson and had him look again at the sympathetic ganglia, and he stained for tyrosine hydroxylase, the precursor of dopamine, and found that the FD patient actually had increased stores of tyrosine hydroxylase as compared to the control, suggesting that with stress, they had the potential of pouring out enormous amounts of dopamine.

And dopamine really has a lot of central effects that we can think of as causing these problems that see,

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the vomiting, the anxiety, and the grimacing that occurs during crisis. It also has peripheral effects on GI motility. It decreases motility. It increases sodium excretion, and dopamine also can be converted peripherally by DBH to norepinephrine and give us hypertension, tachycardia, sweating, and hyperglycemia.

And so there's debate: Is this a GI tract problem or a brain problem? And we think that there's more brain problems and therefore that this is acting like an autonomic seizure and nausea is the aura.

We have also done SPECT studies that have shown that during crisis there's hyperperfusion of areas of the temporal lobe that disappear when the patient is out of crisis.

So we treat for a possible autonomic seizure with GABA-enhancing agents, central adrenergic agonists, and even anticholinergic agonists.

The causes of respiratory problems are aspiration, restrictive lung disease, and chemoreceptor dysfunction. There really is not an increased incidence of wheezing in this population other than based on family history.

However, there is marked chemoreflex sensitivity abnormalities. In particular, they have a diminished response to low oxygen so that when they are challenged

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with a low oxygen situation and you bring the saturation down from 100 to 70, the normal group increases their

minute ventilation, but the FD group does not and this is significantly different. With CO2, it's not as remarkable as with low oxygen.

And what is more dangerous to the patients is that in this particular study, they were brought from 100 percent oxygen and they then were breathing 12 percent oxygen, they did not increase their minute ventilation, as the controls did, but also their blood pressure started to drop and their heart rate did not compensate. So physiological hypoxia, which would be pneumonia, high altitudes, airplane travel, and underwater swimming, basically can be life-threatening for the patients with familial dysautonomia.

Blood pressure. They have postural hypotension without compensatory tachycardia and episodic hypertension. This seems to be a peripheral problem, but this seems to be really induced by central abnormalities. And the peripheral problems, of course, are accentuated by the fact that they're denervated and they cannot mount a normal norepinephrine rise as they go from supine to erect and even then with exercise.

Of course, hypoxic hypotension is a problem, as I just explained, and then they have supine hypertension.

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In a nice study that was done in collaboration with Dr. Goldstein and Dr. Kopin, we noticed that when the patients were supine, their norepinephrine levels actually were elevated, and the only way I can explain that would be possibly this is the effect of increased dopamine being converted to norepinephrine. Interestingly, DOPA levels are high and yet their DHP levels are low.

Neurologic. As they get older, their gait gets worse and there is progression. We're not sure whether this is due to the cardiovascular lability or intrinsic problems with the nervous system. Probably it is intrinsic problems with the nervous system.

Now, I think really what has made our treatment so effective is that there have been two FD centers, one in New York and one in Israel, and that these two centers work very well together. We share our data and therefore we're able to generate sufficient numbers and collaborative studies to be able to see which treatments are effective and which treatments are not. And I could not do that without really the help of my colleague, Dr. Channa Maayan.

There are now 580 FD patients registered in the combined clinic registry. More than 60 percent are surviving. 17 percent are less than 5 years of age, and 35 percent are greater than 20 years of age.

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The effect of just supportive treatment without a gene therapy has been improved survival. In 1965, Brunt and McKusick said that 50 percent of the children would not survive past their 5th birthday. In a repeat analysis of our data, in 1985 we said that 50 percent of the patients were reaching 30 years of age, and in a paper that's coming out this month in the Journal of Pediatrics,

Dr. Maayan and I have shown that 50 percent of our patients are now reaching 40 years of age.

So not only have we improved survival, we feel we have improved the quality of life, improved the appearance of the patients. They seem to have less disability and increase in function, but we hope we can still do better and perhaps with meetings like this, we'll have some insight in how to do that.

Thank you.

(Applause.)

DR. AXELROD: I think the next speaker is Dr. Slaugenhaupt.

DR. SLAUGENHAUPT: I too would like to thank the organizers of this meeting and also thank Felicia for inviting me to speak on our work on FD today.

So I'm charged with introducing to you the FD gene that we recently discovered, and today I'll describe to you the mutations that cause FD and this unique tissue-

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specific splicing defect we see in this disease and also tell you about some studies that are currently underway in my laboratory.

FD is an autosomal recessive disease with a very high carrier frequency of approximately 1 in 30 in the Ashkenazi Jewish population. And in fact, recent large scale population screening using the mutations has confirmed this high frequency. This leads to an incidence of approximately 1 in 3,600 live births. As I said, to date this disease is limited to the Ashkenazi Jewish population, and in 1993, we linked this gene to chromosome 9q31.

Now, in January of last year, two groups identified and published the two mutations that cause FD, ours and a group at Fordham University led by Dr. Berish Rubin. The MGH group started working on FD in the late 1980s under the direction of Jim Gusella by doing family studies to try to identify the genetic locus. This slide basically summarizes the work that we did over the years that culminated with the discovery of the FD gene.

As I mentioned, we linked the gene using family studies in 1993 to chromosome 9, and ultimately we were able to narrow the candidate region to approximately 178 kb by haplotype analysis. 99.5 percent of all FD chromosomes are identical to one another, with only 4

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patients heterozygous for a second haplotype, and this is a remarkably high frequency of a founder mutation in the Ashkenazi Jewish population.

In order to identify candidate genes, we employed exon trapping, cDNA selection and direct gene prediction once we had some sequence, and in order to identify mutations, we used a combination of SSCP and direct sequencing.

Now, a few years ago, we had not identified the FD mutation as yet, and we were approached by Mike Brownstein who said, why don't you just sequence the whole thing? So we began a collaboration with him and generated

the sequence of the entire candidate region. This was before the human genome project sequence was available. At that time, it was approximately 500 kb, and we generated an FD cosmid library from a patient homozygous for the major mutation and we sequenced that from that region and directly compared the control and the FD sequence. Direct comparison of these two sequences identified 152 DNA differences in this region, only one of which was unique to FD, and so we hypothesized that this was, in fact, the pathogenic mutation.

Subsequent to this, we sequenced the four heterozygous patients and identified a second DNA change that was unique to the minor haplotype.

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I'm going to discuss these mutations in detail in a moment, but first I'd like to just introduce you to the FD gene. This slide shows a schematic of the FD candidate region, 178 FD candidate region, which ultimately contained five different genes, one of which is IKBKAP. The name of this gene is IKBKAP, but I'm probably going to say IKAP a lot during this talk because it's easier.

This gene contains 37 exons with the start codon on exon 2. It spans approximately 70 kb of the candidate region. There are two different messages, 4.8 and 5.9 kb, and this is due to differential polyadenylation of the message. It encodes a 1,332 amino acid protein called IKAP, and the message is ubiquitously expressed with the 4.8 kb band being slightly more abundant.

I'm only going to say a few words about the protein IKAP. Jesper Svejstrup is here and his lab has done a lot of work on this protein, and he's speaking later this morning and will hopefully enlighten us.

IKAP was originally isolated as a scaffold protein of the I-kappa B kinase complex in 1998. Subsequent work, however, showed that IKAP is not associated with the IKK's and plays no specific role in NF-kappa B activation. We know that IKAP is homologous to a yeast gene, Elp1 and that Elp1 is a member of the

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Elongator complex, so named because it was isolated in association with RNA polymerase 2 during transcriptional elongation.

Elongator is a six-subunit complex, and in fact, earlier this year, Jesper's lab cloned the human Elongator and has shown that it's also a six-subunit complex.

So recently in the literature, the role of this Elongator complex in transcriptional elongation has been questioned. In fact, there's a new paper suggesting that IKAP plays a role in the regulation of activation of the mammalian stress response via the JNK signaling pathway. So clearly the verdict is still out on exactly what IKAP is doing in this cell, but I think it's likely that it might have multiple roles.

The major FD mutation that is present on over 99.5 percent of all chromosomes is a single base substitution at base pair 6 of intron 20. It's a T to C

change, as I've shown here in pink. And as I mentioned, it's an intron.

When we first identified this change, we didn't see any functional consequence of this DNA change. It was not a coding sequence change, and so therefore, we continued to genotype large numbers of chromosomes in order to build the genetic evidence that this was, in fact, the pathogenic mutation.

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Over the years, we had amassed a large sample of extended FD families that have been completely haplotyped, which afforded us a very large pool of what we referred to as non-FD chromosomes. Basically what this is chromosomes we know not to carry FD by virtue of their haplotype. And as you can see, the genetic evidence here is very strong that, indeed, this DNA change is specific to the FD chromosome.

The second mutation was identified by direct side-by-side exon/intron sequencing of all four heterozygous patients and identified a single base substitution. Again, this is in exon 19 in the coding sequence that causes an arginine to proline substitution. This amino acid substitution is predicted to disrupt a threonine phosphorylation site and in fact, Anderson, et al. showed that R696P IKAP does, indeed, show reduced phosphorylation in cells.

Now, as I mentioned to you, when we first identified the FD mutation, we didn't see any functional consequence. What do I mean by this? Well, if you look here, when we amplified in the message over the region that harbored the mutation from exon 18 to 23, we saw that FD patients expressed the right-sized band. This is the message. We had begun a collaboration with researchers in Claus Scheidereit's group in Germany and had obtained IKAP

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antibodies, and we had, in fact, done a Western blot, which is shown on this slide, that shows that FD patients have IKAP protein. I'll remind you that FD is a recessive disease, so our a priori expectation was that patients would be missing a specific protein.

The key to understanding what was really going on in FD came when we were able to examine FD brain. Through our collaborators at NYU, we were able to obtain two FD postmortem samples, and when we amplified across the region in FD brain, what we saw was in fact this band coming up which is missing exon 20. I'll refer to this from now on as the mutant band. So we see that this band was missing exon 20.

And when we went back and reexamined our lymphoblast and fibroblast cell lines and optimized the PCR conditions, you can see this band coming up in all FD cell lines and not in controls missing exon 20, but clearly there's a preponderance of the normal or wild type message here. You can see from looking at this slide -- and this is actually the figure that was published in our paper -- lymphoblast lines express significantly more normal than mutant, whereas several fibroblast lines show

more equal expression of these.

So, we went on then to develop assays that would allow us to look at both the wild type and the mutant

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messages independently and amplify only those messages. So what I'm showing on this slide is just an example of this in FD cells. If you look at lymphoblasts and fibroblasts, you can see clearly when you do what we call the over-PCR or amplification using primers that flank the exon 20, you see both bands coming up, and by the design of primers, it allows us to amplify only the wild type message and only the mutant message. And you can see in all cases we see the presence of both.

In fact, when we went back with these specific assays to the brain samples that we had originally received, using the wild type assay, we actually saw amplification of wild type IKAP from the brain samples. So originally it appeared that the brain was expressing only mutant, but when we looked specifically for the wild type message, in fact, even though it is slight, it is there.

So this observation led us to suggest that this mutation in FD was manifesting in a tissue-specific manner, and it also serves as the basis for a discrepancy between the two original gene reports. So in the Anderson, et al. paper, they showed us an FD lymphoblast line that was expressing only the mutant band, not the wild type, and so when we first saw this, we were very puzzled. And it led us to hypothesize that perhaps the

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difference in our observations was due to tissue culture techniques, since we were using cultured cells. So we went on to do several different studies, IKAP expression studies, to see if we could address this question.

To date, we've looked at over 81 different FD lymphoblast cell lines and we assay these lines using a combination of methods. We use a densitometric assay in which we generate the integrated density value of each band on ethidium-stained gel using an alpha imager, and we calculate a ratio, a wild type to mutant ratio, of message. And we also use quantitative PCR. And all of the studies that I'll summarize for you today that we've done in my lab over the last year have been verified using quantitative PCR.

So the first thing we did was we went to patients and we collected blood and made new cell lines because clearly the cell lines that we were using in our laboratory had been around for quite a number of years. So those are shown by N here. So we made new cell lines.

We went to Corielle and we ordered some FD cell lines from the Corielle cell repository because these were the source of the lines that were used in the Anderson study. And then we pulled some old cell lines out of the freezer, ones that had been around for a long time and we grew them under the conditions that we grew them all.

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What I'm showing in pink here is the Corielle line that

was presented in an Anderson paper and the same line that was in our freezer for many years, and we pulled them out and grew them. What we saw here was indicative of what we saw in all lymphoblast lines, which was definitely expression of both the wild type and the mutant message.

So we went on to do several other studies, and overall I can tell you that in the lymphoblast lines that we've looked at, the average wild type to mutant IKBKAP ratio is approximately 3 to 1. We do see significant fluctuation, however, between harvests and between cell lines. It appears, however, that the cell lines vary in concert. When we assayed these, they're either all up a little or all down a little, which suggests that there is some uncontrolled variability in the culture or in harvesting that is shared by all cell lines.

We don't see any difference in expression due to common antibiotics that are used in tissue culture, and we also don't see any difference due to switching FBS, fetal bovine serum, percentage. And the reason we did this was because some of the cell lines that we ordered from Corielle suggest that they be grown in 20 percent FBS, and we routinely culture our lymphoblasts in 10 percent FBS. But we didn't see any difference.

One difference we did observe is a significant

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difference. We see a higher wild type to mutant ratio in cultures that are saturated rather than cultures that are in log phase. And we don't have an explanation for this right now, but we could suppose clearly the environment in which cells are growing in saturation is very different than those growing in log phase.

So the bottom line is we consistently see expression of wild type IKAP in all patient cells, and in fact, I can tell you we see expression in all patient tissues of wild type. So to date, the discrepancy between the original reports remains unexplained.

We've gone on to look at IKAP expression in FD tissues. Through our collaborations at NYU and in cooperation with the Brain Bank in Miami and our collaborator at Hadassah, we've collected a large sample of different FD tissues and looked at expression. What I'd like to point out on this slide is just two things.

One is if you look over here, what we see in the neural tissue is a preponderance of the mutant message. As you can see, they do express some wild type message, but they primarily express mutant message. You can see some differences, some equal expression.

But the other thing I'd like to point out is if you look at this lymphoblast line, clearly lymphoblast cell lines are producing a lot of the wild type message,

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more than any of the other tissues.

We did one other thing that I haven't shown on this slide. We went back to patients and we drew many blood samples and we isolated RNA directly from the blood to look at the expression levels in blood and compared them to the lymphoblast cell lines that we had made on the

patients. In fact, what we see is more akin to the fibroblasts. We see roughly equal expression of these two messages in the RNA isolated directly from blood, whereas the cell lines clearly express more wild type. So this is obviously due to something going on in tissue culture, and we don't know exactly what that is right now.

So now we've made what I think is a pretty amazing observation, and that is, that FD cells are not behaving as we would have supposed. Despite the fact that it's a recessive disease, they are able to make some wild type IKAP and wild type normal IKAP protein.

So the next logical question I think is can we somehow increase the amounts or the ratio of wild type to mutant IKAP.

So what we wanted to do was search for chemical compounds that might increase this expression. So the first thing we had to see was if we could use FD cells as the basis for a screening tool. So what we did was looked at parallel cultures. I mentioned that we see fluctuation

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in this ratio between cell lines and, in fact, across harvests, but what you can see here is when you look at parallel cultures, if we take a single cell line and split them into parallel cultures and isolate and screen those cultures, you can see very consistent expression in these parallel cultures of the ratio of mutant to wild type. So even though there's variability within the parallel cultures, it's very consistent, which suggests that we could use these FD cell lines as the basis for screening.

So we took part in the Neurodegeneration Drug Screening Consortium that was sponsored by NINDS earlier this year, and what we and the other consortium members did was screen a panel of drugs. We did a first-pass screen of a panel of 1,040 already FDA-approved drugs and natural compounds to see if we can effect a change in our cells. This month the meeting report will appear in Trends in Neurosciences.

We completed the initial screen and what we are doing now is doing the follow-up studies. We took the top 20 compounds that effected a change in terms of increasing wild type to mutant because it is our hope that these could potentially lead to therapies for FD. We're also examining, however, the bottom 20, I guess you would call it, the compounds that increased expression of the mutant because these may be interesting mechanistically.

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So in our follow-up screen, we did a first-pass screen using a single cell line for the initial pass. So what we're doing is we're using multiple cell lines, different cell types, and calculating dose curves and things like this. And these studies are currently underway. What we hope to do is to be able to use these to then test these compounds that we identified in animal models that are currently under construction.

So I'm going to switch gears a little bit here now and take you back to your early basic genetics days and talk about some other studies that are underway in my

laboratory in collaboration with Robin Reed at Harvard Medical School where we're looking at the FD splice defect itself.

So this is the consensus splice donor site. So you have to think back in your brain to when you learned about splicing in genetics 101. This is the consensus site, and what you see here is the FD mutation, which occurs at base pair 6 here. Although T is the preferred base at this position, this is not very well conserved, and when we initially looked at this, we didn't think that this would have such a pathological effect. You could see that the FD change, which is a C here, still occurs in 17 percent of all exon splice donor sites. In fact, there are three other exons in the IKAP gene that have this same

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"mutant" sequence, or FD sequence, at their splice donor site.

So what happens in splicing? What I've shown here is just the exons 19-20, and this is the sequence of the spliced donor site for exon 20. In normal splicing or in splicing in general, it's accomplished by the spliceosome. The spliceosome is a complicated structure that's characterized and it contains 5 snRNPs and about 50 to 100 different proteins. We don't know exactly what all these proteins are, and clearly the role of tissue-specific alternative splicing is becoming more and more evident in the literature, and what different proteins associate with the spliceosome in different tissues may very well govern what exons are spliced and how genes are spliced.

So anyway, in normal splicing what you have is U1 snRNP comes in and binds the splice donor site. U2 comes in and binds at the branch point, and then the other snRNP complex comes in. The spliceosome assembles. You have lariat formation and the splicing together of two exons.

Clearly the FD mutation is effecting a change here so that exon 20 is in some cases skipped. It's skipped more often in brain tissue, in neural tissue than it is clearly in lymphoblast cell lines. They have a

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pretty good mechanism for compensating for this because generally exon 20 is included. So exon 20 here is skipped.

So our question really is what influences the ability for this to splice correctly?

So if you look at the splice sites around this region, what I've shown on this slide is the sequence of the splice acceptor and splice donor sites for exon 19, 20, and 21. These numbers here are bits, which is basically just an expression of information content of the strength of the donor and acceptor sites.

And as a means of reference, because until recently I had no idea what a bit was either, I've put this on the slide. In IKAP, if you look at all 37 exons in IKAP, the acceptor site strength ranges from minus 2.5 to about 15.8, with an average of 9.47, and the donor site

strength ranges from 3.2 to 13, with an average of 8.94.
So you can see that if you look at exon 19, it has an incredibly weak -- in fact, the weakest in the gene -- splice acceptor site. However, we know exon 19 doesn't have any problems with splicing.
If you look here, the FD mutation drops the bits, or the information, for the splice donor site from 10.1 to 8.7, but still not a drastic change or a drastic reduction in the information content in this splice site.

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So clearly there's more going on in splicing than just this strength or weakness of the splice sites.
So in this lovely figure that I lifted from a review, I'm going to talk to you a minute about exonic splice enhancers. As I said, the splicing process is very complicated, and the spliceosome is made of many different proteins. And there's a class of proteins called SR proteins that are known to contribute to splicing. And in fact, through knock-out studies, it's been shown that these SR proteins are not only necessary for splicing, but necessary for development.
So there are sequences called exonic splice enhancers that are able to bind these SR proteins, and the SR proteins serve as splice enhancers, which then serve to recruit the splicing complexes and enhance the splicing of particular exons.
So for the class of SR proteins, now these exonic splice enhancers, there are sequences that have been identified using various methods. It is now believed that all exons contain exonic splice enhancers, multiple exonic splice enhancers it is likely. I need to point out, though, that these are just predicted based on binding sequence, and certainly, like I said, it's a very complex process. There are also exonic splice silencers. So until you look at a specific region experimentally,

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it's very difficult to say exactly what's going on. But looking at this slide gives us an idea.
If you look at exon 19, which has the worst splice acceptor site in all of IKBKAP, and then you do an exonic splice enhancer prediction, you see that there are 23 different exonic splice enhancers predicted to lie within this exon. If you look at exon 21, which has very strong splice sites, there are only 6. But if you look at exon 20, which has pretty weak acceptor sites, you see that there's only 4 predicted splice sits. In fact, if you look at all of the IKAP gene, at all exons with weak splice sites, the average number of predicted exonic splice enhancers is 16.8. So 4 is pretty low.
So what we begin to see emerging here is potentially a picture of a weakly splicing area and perhaps the FD mutation is just the straw that broke the camel's back, that this area is weak in terms of splicing and that this mutation introduced here has a drastic effect on splicing; whereas, a similar mutation at base pair 6 of the splice donor site in another exon may not, in fact, be pathogenic at all.

So in the splicing studies that we're carrying out, we've in fact shown this. By using in vitro and in vivo splicing assays using mini-gene constructs, we've shown that the wild type exon 20 does, in fact, splice

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poorly. So forget even FD mutation right now. This is just the wild type exon 20 splices poorly. Mini-gene constructs that contain the complete genomic sequence from exon 19 to 22 routinely skip exon 20.

So we're currently looking at methods to strengthen exon 20 by adding certain enhancers sequences or by introducing exonic splice enhancer sequences via site-directed mutagenesis to try to increase the strength to see if we could potentially override the FD mutation by strengthening the exon itself.

And our other question is, can we alter the amounts of specific SR proteins or other splicing factors and therefore increase wild type IKBKAP expression? So by understanding the mechanism behind the defective splicing going on in FD, we actually may be able to alter the wild type to mutant ratio.

So in summary, I would just like to say that I think that although FD is a devastating disorder and the gene was a pain to find and it took us a lot of years, I think that in the end, we're relatively lucky because typically when you identify a gene for a genetic disease, you're left but with one route in order to identify therapy for the patients, which clearly is the goal of everyone in this room today, and that is identifying what is the role of IKAP in the cell. Clearly this is a very

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important question. We have to identify the role. We need to understand IKAP, know what it interacts with, and this will be accomplished by cellular studies, by the construction of mouse models, and may eventually someday, through the understanding of IKAP, lead to therapy.

But we have two other mechanisms. If we can understand the mechanism behind this aberrant splicing -- forget right now that the gene is IKAP -- what if we can understand this mechanism and somehow effect a change on the splicing? This may also lead to therapies.

And lastly, forget mechanism. In the quick and dirty way, our drug screen may, in fact, identify drugs that increase the wild type to mutant ratio. So we don't even have to understand the mechanism behind this right now. We could go on and understand that later.

So I say I think we're rather lucky in that all three of these avenues are clearly being pursued by myself and many other people in this room. I think that through these studies, our hope of identifying therapy for FD patients may, in fact, be realized.

In closing, I have to thank the FD team. I'm not going to list everybody. They're from all over the world, and without all of these people and our close collaboration, we couldn't have accomplished any of this. I'd like to acknowledge the funding agencies that support

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the work in my laboratory.
Thank you.
(Applause.)
DR. AXELROD: Thank you, Dr. Slaugenhaupt.
Our next speaker will be the professor, Dr. Hilz. We're talking about eventually treating FD patients, but without a way of assessing what has happened before, we will not be able to judge what will happen in the future. So Professor Hilz will give us an insight as to clinical assessment of our patients.
DR. HILZ: Thank you, Felicia. I'd also like to thank the organizers for giving me the opportunity to present some of the work we have done over the last 10 years.
When I first started to work with Dr. Axelrod, I looked at the patients from the perspective of a conventional neurophysiologist and I rapidly learned that the given neuropathology, which you have already been introduced to, does not allow us to use conventional techniques.
You have seen this slide that indicates that it is primarily the unmyelinated fibers that are largely reduced. In the peripheral nerves, you barely find any of the unmyelinated fibers in the sural nerve biopsy. And therefore, conventional techniques of nerve conduction

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studies don't take us very far. What we saw is this double-peaked response in the motor conduction velocity or a double peak in the sensory nerve action potential, a finding that's been already described in '67 that does not allow us to really quantify severity of the pathology. It might be helpful to assess disease progression over decades, but it doesn't assess the current situation of the patient.
In order to quantify peripheral nerve function, we had to use psychophysical methods of quantitative thermal testing. This procedure depends on the patient cooperation. A small Peltier element, also called the Thermode, is attached to the patient's skin and with quantitative stimuli of cold or of warm or heat, we can then judge the function of thinly myelinated A delta fibers that mediate cold or unmyelinated C fibers that mediate heat or warmth perception.
There are various algorithms that can be used. We used the simple method of limits because it is straightforward and allows us to test children. After calibration, the Thermode delivers a warm stimulus. As soon as the patient perceives the stimulus, he stops further stimulation. The stimulation returns to baseline, and this is repeated several times. We then take the warm or cold threshold as the average of the peak-to-baseline

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differences, and that gives us quite a good impression of A delta and C fiber function.
The question was does that work in the young children. There were no data available in children, so we had to first establish a database that clearly showed that

even in very young children, the technique provides reliable information regarding small fiber function.

What we saw is highly abnormal warm and cold perception in the FD patient. On average, cold and warm thresholds were up to 8 to 10 times higher than in the control group regardless of the tested body site. We tested up to six different body sites.

Dr. Axelrod already pointed out that the reduction of peripheral small nerve fibers is very similar to the reduction of sympathetic neurons, of neurons in the intermediolateral column or in cervical and thoracic ganglia. And she also pointed out that there is a high rate of unexplained death, and so far, there are no risk predictors that might indicate whether an individual patient is at specific risk.

So we were wondering whether a combination of evaluation of the peripheral nerve function and autonomic function might help us identify patients at risk, and we did a correlation of peripheral small fiber function as assessed with a temperature perception procedure and of

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cardiac parameters, and for that we used the corrected QT interval.

What we actually saw is that there is a correlation between impaired small fiber function as expressed by increased thermal perception thresholds and impaired autonomic control, in this case expressed by QTc prolongation. What we found was that 4 of our patients in a group out of 20 who had abnormal cardiac parameters also had severely impaired peripheral small fiber function. Probably more importantly, 3 of the patients who had severely abnormal peripheral nerve function experienced cardiac arrest within one year after the study.

Of course, this is just a surrogate marker, and to better understand risk factors, we conducted a variety of studies evaluating the peripheral cardiovascular and the central autonomic nervous system. And I cannot go into all these tests. We did a cold face, cold pressor, Valsalva test. We challenged the orthostatic control. We conducted baro- and chemoreflex studies and tested vasomotor control and lately conducted a gastrointestinal challenge test.

I'd just like to point out that a trivial test like the cold face test allows us to gain further insight into the pathology. This is a test where you stimulate the trigeminal area with ice and that activates in the

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brainstem a response to the peripheral sympathetic sudomotor system with vasoconstriction and blood pressure increase, but simultaneously activates in the brainstem the cardiovagal, the parasympathetic system and induces a bradycardia, and this bradycardia is considered to be independent from the baroreflex.

So in the patients, we did not see with this test any increase of blood pressure as we saw in the controls. We did not see any reflex bradycardia, and more importantly, heart rate variability didn't change at all

while it increased in the controls, and clearly showed an enhanced parasympathetic function. And this parasympathetic function in the controls, here the black bars, during the cold stimulation went up significantly. There was, with spectral analysis of heart rate variability, much more parasympathetically mediated, high frequency modulation of heart rate, but no change at all in the patients. So with this simple test, we were able to demonstrate that there is efferent parasympathetic dysfunction which is most likely due to a central parasympathetic deficiency in the patients.

The parasympathetic deficiency was also confirmed in another fairly simple test, the Valsalva test. The Valsalva test induces increase of heart rate during excretory strain and a reflex bradycardia after

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release of the strain. Normally this reflex bradycardia is fairly pronounced and is due to a baroreflex-activated cardiovagal function. In the FD patients, there was no such response. The Valsalva ratio was abnormal.

But the test was even more important because it allowed us to assess cerebral auto regulation. We monitored it with transcranial Doppler sonography, the changes of cerebral blood flow velocity. And you see in the early phase of the maneuver, an increase of blood pressure both in patients and controls, and then an early dip during strain which then turns into an increase of blood pressure. And after release of the strain, there is even a blood pressure overshoot.

Now, for a cerebral blood flow velocity, this increase should be steeper and faster than for blood pressure, and the same is true for the increase after release. The overshoot increase of cerebral blood flow velocity should be higher than the overshoot for blood pressure. That indicates normal autoregulation. In the patients you see the response is very sluggish. It's slower than the response of the blood pressure for both phases. So we saw impaired cerebral autoregulation, another new insight, and we think this is due to an increased rigidity of the intracerebral resistance vessels, most likely due to the longstanding, excessive

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hypertension when the patients are supine, meaning when they're asleep.

This was also confirmed in a simple head up tilt study where we monitored heart rate, heart rate variability, blood pressure and again cerebral blood flow and cerebrovascular resistance. Of course, we saw, as expected, orthostatic hypertension with a prominent drop of blood pressure and absence of heart rate increase, while in the controls there was some reflex tachycardia. Again, with heart rate variability assessment, we saw that in the controls, they increased their overall autonomic modulation in comparison to the supine modulation. And they enhanced their sympathetically mediated, so-called low frequency modulation of heart rate, but in the patients there was no increase of the overall modulation

and no change of the sympathetic or parasympathetic activity.

More importantly, we saw a discrepancy between the peripheral drop of blood pressure due to lack of peripheral sympathetic activity and an unexpected secondary increase of cerebral blood flow velocity which contrasted with the slight drop of cerebral blood flow velocity in the controls. This again indicates impaired cerebral autoregulation and might be due to a preserved central sympathetic activity or discharge of neural

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humoral factors. It seems to be due to increased rigidity of the resistance vessels and that again might result from the longstanding changes between permanent orthostatic hypotension and nocturnal hypertension.

During orthostasis, the patients are not only unable to modulate their autonomic control, but they, moreover, cannot even enhance their cardiac stroke volume. While the cardiac output increases significantly upon orthostatic challenge in control persons, there's even a drop of cardiac output in the patients, and the patients cannot compensate the shift of the fluid to the lower extremities by increasing peripheral vasoconstriction, but the total peripheral resistance that goes up significantly in the controls only increases insignificantly and sluggishly in the patients.

So given the lack of central and peripheral sympathetic and parasympathetic modulation, the lack of adaptation of stroke volume or peripheral resistance, we were wondering how do the patients still increase their blood pressure and their autonomic output during this autonomic crisis, how do they generate sweating, peripheral vasoconstriction, mottling of skin. Is that due to denervation or hypersensitivity or is it due to a central autonomic dysregulation?

To better understand this, we did a

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microdialysis study. We inserted a micro-catheter into the superficial skin layers, and this catheter was perfused with electrolyte solution, and then we added some drugs to the solution. We were able to measure the drug-induced changes of skin blood flow by means of laser Doppler flowmetry, the changes of humidity output, and of course, we were able to measure the amount of plasma extravasation.

For vasoconstriction, we added norepinephrine and we analyzed changes of skin blood flow, plasma extravasation, and the area of skin blanching due to the vasoconstrictor substance.

For sudomotor function, we added acetylcholine and measured sweat output by means of quantitative sudomotor axon reflex testing and transepidermal water loss measurement.

We didn't see any major changes of the axon reflex sweating, no difference between patients and controls, but we saw hypersensitivity to norepinephrine. There was an earlier start of vasoconstriction in the

patients, here the dotted line, and the reduction of plasma extravasation with norepinephrine just lasted longer than in the control group. Moreover, the area of blanching was greater than in the controls.

So there seems to be a peripheral denervation

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hypersensitivity, and that might account for some of the hypertension seen during crisis. But there must also be some central component as suggested by the normal sweat responses.

To better understand the central mechanisms, we did a selective stimulation of the response of the sympathetic branch and the parasympathetic branch of the autonomic nervous system by activating the baroreflex with suction-neck chamber. We applied a sinusoidal vacuum that stimulates the carotid baroreceptors and we used a pressure of minus 30 millimeters mercury. This procedure, developed by Luciano Bernardi, allows us to quantify the response of the sympathetic system at the level of the heart and blood vessels. If you apply this stimulus at a frequency of 6 cycles per minute, that's .1 hertz, and if you double the frequency to 12 cycles, the sympathetic cannot follow anymore and the response is only a parasympathetically mediated response at the level of the heart. Respiration is kept constant to avoid interferences of the respiratory influences.

So here in one control person, you see the respiratory peak due to the paced breathing. You see the same peak in the patient, and now with sympathetic stimulation, or a .1 hertz stimulation, we have a clear-cut sympathetic response in the control, but no such

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response in the patient.

The same held true for the whole group. In the controls we saw a clear-cut increase of sympathetic output, but no such response in the patients. And for the parasympathetic, .2 hertz stimulation, or the parasympathetic response to the .2 hertz stimulation, again in the controls we see a significant parasympathetic activity, but no increase in the patients. So they're unable to enhance their sympathetic and parasympathetic responses.

The same was confirmed when we looked at systolic and diastolic blood pressure changes during .1 hertz stimulation in controls and in patients. Again, in the patients, no sympathetic response.

Now, given the fact that the baroreflex is compromised and knowing that the patients have respiratory problems -- they have breath-holding spells, sleep apnea. They can, as Dr. Axelrod already pointed out, not trust their respiratory pattern to environmental needs when they're in an airplane, or when they have respiratory infection, they cannot trust the frequency or the volume. And we also know that many of the causes of death are due to pulmonary or unexplained cardiovascular events.

This is extremely important if we see this in combination with the impaired baroreflex function. Somers

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and co-workers showed that if the chemoreflex is activated, but the baroreflex doesn't function, this could end up in an unopposed bradycardia and asystole, and they call that a cardiovascular catastrophe. And those working with the FD patients are well familiar with this catastrophe.

What happens was tested in a chemoreflex study. Dr. Axelrod already showed some of those data. We did various stimulations. I'll just show you the closed circuitry breathing technique. The patient breathes his or her own air, which is sampled in a bucket, and if you do that, of course you enhance CO2. CO2 can be filtered. Then you only have progressive hypoxia, or if you supplement through the inspiratory port oxygen, you have progressive hypercapnia, if you do not scavenge CO2, of course.

In the patients and controls, we were able to decrease oxygenation to a similar extent. Dr. Axelrod already mentioned the controls quickly enhance their respiratory frequency and tidal volume. The patients do not change their frequency or their tidal volume.

Moreover, patients cannot increase their heart rate. You see in the controls RR interval decreases, meaning the heart rate goes up. In the patients, we have the opposite, RR interval increases, patients go into

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bradycardia.

In the periphery, skin blood flow in a control is reduced indicating vasoconstriction and, of course, secondary increase of blood pressure. In the patients, we see a paradoxic peripheral vasodilatation and, of course, a drop in blood pressure.

So to summarize this, there is a reduced sensitivity of the chemoreflex to hypoxia. Moreover, if there is hypoxia, we see a paradoxical response: a blood pressure drop, vasodilatation, bradycardia. This is not only dangerous when the patients are in situations of low oxygenation, like being in the mountains in high altitude or in an airplane, but even during daily life, there might be complications because -- I haven't shown that, but the chemoreflex sensitivity -- Dr. Axelrod showed one of the slides -- is rather normal towards hypercapnia, but the resting CO2 levels of the patients are higher.

What happens when they are stressed, when they are excited and they go into simple hyperventilation could be rather critical. They reduce their CO2 levels. This might take away the respiratory stimulus and lead the patients into a respiratory pause, which then secondarily, of course, induces deoxygenation with all the consequences of hypoxia, bradycardia, vasodilatation, blood pressure decrease, and the cardiovascular fatality.

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This is an example of one of the patients we studied. You can see that here the patient is asked to hyperventilate, so the respiratory drive is well preserved. CO2 goes down from 36 to 30. Now the patient

suddenly stops to breathe. Here the patient went into tachycardia, shortening of RR intervals. As soon as respiration stops, this tachycardia turns into bradycardia which would progress. Here you see the secondary deoxygenation. Oxygen goes down. And now Dr. Axelrod just squeezed the patient and said, come on, take a breath, and this stabilizes the situation again. But what if Dr. Axelrod or Dr. Maayan is not around at night? Then the patients might well go into unopposed asystole, and this might lead to a fatality.

Let me end by saying we know FD is a progressive disease. Still, the treatment efforts, the clinical risk stratification like fundoplication, gastrostomy, have improved the life expectancy significantly, as Dr. Axelrod has indicated. And there is a chance to further improve it.

I am afraid -- as a clinician I may say that -- that those patients who taught us about the disease and who taught us about the gene are not likely to benefit from gene therapy within the next one or two years, but their disease and their problems are still progressing.

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Therefore, I believe that it is essential to further improve the understanding of the peripheral and central autonomic pathophysiology, particularly of the changes that happen during daytime and during nighttime, and this might lead us to further therapeutical options that hopefully help to improve the quality of the patients and their lives.

Thank you.

(Applause.)

DR. GWINN-HARDY: Thanks a lot. I'm going to, in the interest of time, suggest that we skip the break and move right on into the next talk. Of course, I want people to feel free to get up and get snacks and go to the bathroom as they need to. But the most important thing I think is that we don't sacrifice the discussion because I know that I for one have a lot of questions to ask the speakers. So let's move right on into the next talk then, if that's all right.

Felicia, do you want to briefly introduce the next speaker? Thank you.

DR. AXELROD: Our next speaker is Dr. Gail Sonenshein. She is the head of the Scientific Advisory Board of the Dysautonomia Foundation, and she is going to talk about future directions for familial dysautonomia, sort of an overview and maybe a wish list.

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DR. SONENSHEIN: I too want to thank the organizers and the NIH for sponsoring this meeting.

I think following the successful identification of the involvement of the IKAP protein in FD by two groups, it led the Scientific Advisory Board to sponsor a brainstorming session in New York last year, and that Scientific Advisory Board was being headed at that point by Kurt Hirschhorn, and actually I was invited to participate. Little did I know what would happen as a

result of that invitation.

So from that brainstorming session, what came out was an RFA for what we viewed at the time were the research directions that we would like to forward in the quest of understanding more of the disease and possibly arriving at a treatment. So this summarizes the research directions that came out of that.

One was to elucidate the function of the IKAP protein and to identify interacting proteins.

Next was to determine the loss of IKAP function and how it affects cellular processes and survival.

To elucidate the control of tissue-specific splicing in gene expression and potential to mediate correction of splicing defects.

To elucidate the role of IKAP in the development and maintenance of the autonomic and sensory nervous

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system.

And to determine the effects of IKAP knock-in and knock-out mutations using mouse or other animal models.

And lastly, but certainly not least, to search for novel therapeutic strategies for FD. We've already heard from Sue, and we're going to hear from Drs. Svejstrup and Xu who are all being actually funded by the foundation.

So I think the key issue still to date is exactly what does IKAP do, and I think that Sue sort of summarized a little bit, but IKAP was originally identified as a component of the IKK complex which is involved in activation of NF-kappa B which is actually the transcription factor that I work on.

It turned out that that appears to be a false lead. Many people now have repurified the IKK, and when I called members of the NF-kappa B group who work on it, I either got polite or rude or sort of aggressive answers, telling me that they have, of course, wasted a lot of their time. IKAP is not in the IKK complex.

Fortunately, work by Dr. Jasper has shown that IKAP is part of a complex which was first identified in yeast. It appears to be involved in transcriptional elongation control. The whole complex is called

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Elongator. The yeast homologue is Elp1, and as Sue mentioned, Elongator has now been found in mammalian cells both by Dr. Svejstrup's group and also by Danny Reinberg's group.

I think there are some issues related to this about the localization of the IKAP and the Elongator complex, and I'll show you a few reasons why I think there's still an issue with this in just a few seconds.

Microarray data was performed on the yeast by Krogan and Greenblatt, and they identified a subset of genes that were affected and many of them are, in fact, involved in metabolism. But there's still a lot to learn about the function of IKAP, and hopefully we'll get some more microarray data shortly to help us with this in human

cells.

I think since Dr. Svejstrup is really going to talk about the Elongator, I just want to point out that there is a new paper that has just been published on another role, a potential functional role of IKAP. I don't think that the fact that we have two potential functions means that one is right and one is wrong. I think that in many cases in eukaryotic biology, you can have a component of one complex actually play another functional role. So it remains to be seen what's really happening, and obviously the next six months will probably

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be very helpful in elucidating some of this.

The other functional role of IKAP is involving it in stress responses, and the evidence indicates that IKAP can interact with a specific kinase called the c-Jun kinase, or JNK, and it is interacting via its C terminus, IKAP C terminus. JNK not only targets IKAP, but IKAP potentiates the kinase activity. Actually I didn't realize that the senior author of this would be here, Dr. Kallunki. I think this is sufficiently important for people to know about this data that I took some of the figures. That's the power of the Adobe Acrobat. I took some of the figures her paper illustrate some of the points.

So it was a yeast two-hybrid screen that identified the interaction between JNK and IKAP, but that's actually not considered stringent enough proof. So the group showed by co-immunoprecipitation that if you precipitate JNK, you find IKAP. So that clearly indicates an IKAP-JNK association.

This is an immunohistochemistry looking at IKAP and JNK localization within the cell, and this is the issue. IKAP complex appears to be located not exclusively because Danny Reinberg's group purified the Elongator complex actually from the nucleus of HeLa cells. So there's clearly presence in the nucleus, but a lot of it

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appears to be in the cytoplasm.

This is the JNK staining, and here is the overlay. Actually it's not yellow. It's orange, so unless I'm reading this wrong -- and fortunately, Dr. Kallunki is here so she could comment if I'm misstating. But it looks like it's not a total overlap.

These are further analyses showing the presence of IKAP using a tagged IKAP, and what you can see, again here's one tag or another tag. It's predominantly in the cytoplasm, and that's actually also been shown in Danny Reinberg's paper where he actually also looked at making in HeLa cells with an antibody he prepared and also finds it predominantly in the cytoplasm. It doesn't mean it cannot move to the nucleus under certain stimuli, and that's actually one of the issues that may become important to look at.

This is evidence. I didn't take the figure that shows that JNK actually can phosphorylate IKAP, but that was done in vitro. These are analyses showing that the

signaling cascade by JNK is affected by IKAP in the sense that it potentiates the ability of the kinase to function. So this is downstream. There's a full cascade that's been worked out, and this is one of the kinases that's upstream. What you can see is this is the kinase activity in the absence of IKAP, and this is the presence of IKAP.

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And you see there's enhanced activity.

JNK kinase is what responds in stress to certain stimuli, UV, TNF, and EGF. And this is the JNK activity in the absence of IKAP in all these responses. They are stimulated in the presence of IKAP.

So what this implicates is the IKAP protein may be playing an important role in modulating JNK activity, that this kinase activity is the major stress response.

I think what's even more interesting was the fact that when they did the yeast two-hybrid work, the IKAP protein that they pulled out was actually a portion of the C terminus and that's the part that interacts. So if you look at the IKAP protein that would be produced by the FD as a result of the mutation, what you see is it's not containing that part. So if the role of IKAP is to, in some way, affect the JNK response, then the FD kids would have an altered response.

I apologize for taking so much time. Dr. Kallunki obviously should have, I guess, been here to present it.

So what do we need? I think what we need is mouse models. We have to able to elucidate the role of IKAP and the effects of the mutations on the nervous system. One of the things that we'll be able to do from knock-in mice, with both major and minor mutations, would

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be to get models and cells to elucidate both mechanisms, as well as to test potential treatments for therapy.

We're also looking at making knock-out mice with the Cre locks which would allow us to put it into any developmental system or actually in any cell type depending upon the promoter. And all of this work is being funded, and Dr. Xu is going to talk today.

So where do we go from here? I think obviously there are a lot of things we need to do. One is to identify potential therapeutic drugs that promote the splicing. This would obviously be in many ways we presume for the major mutation, although we don't know. Maybe pushing a splicing might be of help in other ways as well. The FD Foundation is funding work by Jim Gusella in this aspect.

We're also interested in elucidating the role of IKAP in neural cell survival and to identify potential targets of IKAP or the total Elongator. The targets may be alternative ways of treating patients because these are essential pharmacologic targets as well as the IKAP itself. So that's another way of looking at the issue. And the foundation is funding Math Cuajungco to look at this.

Now there is stuff we're just starting to think

about as a result of some of the new work that's being done, but also some of the old work. We'd obviously like to look at factors that interact with IKAP, specifically also neural cells, because the JNK signaling kinase pathway, and actually almost every signaling pathway, shows some cell type specificity. So I work on NF-kappa B. NF-kappa B in almost all cells protects from cell death by any number of agents, but it turns out in neural cells it actually sometimes promotes death. So there are cell type specific effects even on the most common signaling cascades that exist. So we really need to look at the effect of IKAP in neural cell survival and particularly the neural cells that we're dealing with here.

We also would obviously like to look at yeast model systems because they're easier to work with, and we want to use both biochemical and genetic tests. There are vectors, besides these new, fancy vectors that would allow you to clone out within the space of a short time, that have two tags on them that allow you to go from unpure to a million-fold purification in basically a single step.

We could look at what are the factors interacting with IKAP in multiple cell types, look in specific cell types. There are new yeast methods. The Elp1 is not lethal. But the question is could you make a double mutant or find something that, instead of complementing the Elp1, would actually promote the disease progression, promote the death of the cell. And that's a very common way for looking at getting two members in the same complex and that would allow you to maybe sort out better what Elp1 IKAP is doing.

We'd like to look at the control of IKAP expression because we'd like to see what the effects are on the level of IKAP. What affects the level? Does it get altered?

I have to say that I tried not to work on IKAP now, but when it was first discovered that IKAP was involved in FD, since I work on NF-kappa B, we quickly did a few experiments. This is unpublished data that was done by a former post doctoral fellow in my lab. This has been done in vascular smooth muscle cells, which we work on. It was also done in NIH toto3 cells. The result in the red -- if you look at red staining, this is the result of TNF-alpha treatment which is a stress response. What we saw was in the absence of TNF, low levels, and in the presence of TNF, much higher staining.

Originally we didn't do anything with this because some of the original work implied that IKAP was only in the cytoplasm, and we said, oh, we must have a mistake here. We put it in the drawer and it wasn't until the recent paper on JNK came out that I said, wait a minute, let's go back and take a look. So it is possible that things can modulate and then affect the actual response of the IKAP.

Not only do we want to look at level, but we want to look at cellular localization. Does IKAP always sit in the cytoplasm, or is there something that will induce it? Maybe there's a combined stress response that will move IKAP and the Elongator maybe more into the nucleus where it might have more of a functional role in controlling transcription.

There's obviously evidence for post-transcriptional modification. There was worked that Berish published originally that the point mutation affects the phosphorylation of IKAP protein. But there's also the data that just came out that JNK kinase phosphorylates IKAP. How does this affect its functional role? This could be a very critical issue.

And lastly, I just want to mention that there are viruses that affect JNK kinase and signaling. We have work that's totally unrelated to this. In fact, I never work on JNK. I don't work on JNK, and then all of a sudden, it's surrounding me. We work on cytomegalovirus. There's a protein IE1 of cytomegalo which promotes growth, vascular smooth muscle cells, and other cell types. It turns out it induces JNK kinase. So do other viruses

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induce it? Rous sarcoma virus has been implicated. Does that have some role in affecting the JNK kinase signaling pathway?

Lastly and not least now, the question is what are the effects of IKAP on stress responses in neural cells and obviously then in vivo once the mouse models become available.

Lastly, as I was talking to people here, some people had seen the JNK kinase paper. Some people had not, and I think that we need to have better communication, at least as far as what's happening. I think that the more rapidly things get communicated, the better it's going to be. I was going to propose that I would be happy to make an e-mail list. If we make e-mail list, any paper that comes out can be circulated. If you want to submit a preprint, you can decide. If you don't want to submit it before it's accepted, fine. If you want to submit it after it's accepted, fine. But if we make an e-mail list, with a press of a button everybody is going to have those papers. We could make a list. I'm happy to put out a pad out in the hallway, and anybody who wants to be on the e-mail list, let's make one so that everybody gets the information as quickly as possible. The more information passed, the better we're all going to be.

My only agenda in this is to help the kids.

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Many people don't know but actually my nephew has FD which is the reason why I'm here and the fact that my sister-in-law Jennifer is so persuasive.

(Laughter.)

DR. SONENSHEIN: And I'll stop there. Thank you.

(Applause.)

DR. AXELROD: Thank you, Gail. I think really

the e-mail list is an excellent idea. I think if you put a pad outside, I think a lot of people will put their e-mail address on it.

Now we're all really waiting to hear the next presenter, and that's going to be what is IKAP and speculations on its role in development, maintenance, and survival of autonomic and sensory neurons by Dr. Svejstrup.

DR. GWINN-HARDY: Why don't we just take a 10-minute break while we're setting up the computer because there's no reason for you guys to sit in here while we're doing this anyway, and a lot of people probably have to go to the bathroom. But come back in 10 minutes.

(Recess.)

DR. GWINN-HARDY: I'd like to introduce our next speaker who is Dr. Svejstrup, who is going to talk about what is IKAP and speculations on its role in hte

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development, maintenance, and survival of autonomic and sensory neurons. Dr. Svejstrup, thank you very much.

(Applause.)

DR. SVEJSTRUP: I think I'll get started. I too would like to thank the organizers for this opportunity to come and present some of our work to you today.

I want to start out by emphasizing that I am not in any way an expert on familial dysautonomia. As a matter of fact, just two years ago I had never heard about FD before, and now as you've seen on some of the previous slides, we are part of the important battle against this disease in that we are trying our best to understand the molecular function of the IKAP protein.

Our starting point into all this is not work with patients. It's not even work in human or mammalian cells, but actually in the lowly eukaryote Saccharomyces cerevisiae, or baker's yeast, where we try to understand how genes are regulated and transcribed with a mixture of biochemical and genetic techniques.

During the course of our work in yeast, we have realized that even though this is a low eukaryote, all the major or the very important basic cellular processes that govern the cell remain highly conserved in evolution and that we can, therefore, actually very often learn quite a bit about human disease by investigating life in yeast.

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This first slide shows you the focal point for all work in my lab, namely, the elongating form of RNA polymerase II which, of course as you all know, transcribes all protein encoding genes in eukaryotes. We are interested in the question of how this polymerase, as it leaves the promoter and travels to coding regions, so to say, deals with obstacles such as chromatin structure and also DNA damages or DNA lesions such as those that are produced by UV lights and therefore are a common feature of all cells.

Now, in this talk today, I will not be talking about DNA damage at all, but I will be talking about how we think the IKAP protein has a connection to how

elongating polymerase might be able to deal with the obstacle that chromatin is during transcription.

From the outset, we knew quite a bit about the polymerase form, the form of RNA polymerase II, that is involved in initiating the transcript. Because the majority of research into regulation of gene transcription is at this level, because the majority of regulation of transcription is actually at the initiation level, we knew quite a bit about the initiating polymerase and about the basal factors that are required for it to recognize a promoter and get transcription started. But we knew much, much less about the polymerase form that is involved in

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elongation.

So basically that was our starting point. Are there other factors that are specifically able to associate with the polymerase when it is engaged in transcription?

So what we did was to make simple biochemical assay by which we caught in the act the polymerase as it was transcribing DNA. I shall not bore you with all the biochemical detail of how we did this, but just show you the endpoint of this endeavor, namely the isolation of a novel, very, very large protein complex containing RNA polymerase II, here designated by its Rpb subunits, and a number of novel proteins, only some of which we have so far been able to isolate and identify by mass SPECT analysis, a very powerful tool, by which you can basically cut out the bands in a protein gel like this and identify them. And because of all the genomes, not the least that of yeast, have been fully sequenced, we immediately know what the gene that encodes this protein is.

As you will see later, we've been able to now identify all the six subunits of the human Elongator complex and thereby the five proteins that interact in our hands. The strongest were the IKAP protein.

So this basically is what we call the elongating form of RNA polymerase II even though we have to admit

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that the definition is somewhat loose.

Now the p150, p90, and p60 subunits, which is what we identified first, turned out to actually in themselves form a complex that then interacts directly with RNA polymerase II. And we now know that the Elongator complex, which you've heard mentioned a few times, actually is six subunits: Elp1, Elp2, Elp3, Elp4, Elp5, and Elp6. And we can purify those from both yeast and mammalian cells fairly rapidly because we developed very powerful tools for this endeavor.

What do we know about these genes? Now I'm confining myself to yeast. Well, of course, of interest to this audience is the fact that Elp1, also called IKI3 is the yeast homologue of IKAP. But actually of most importance for us in terms of our quest to understand how polymerase deals with obstacles is defining at Elp3 -- it's a very highly conserved -- you'll see that later -- histone acetyltransferase. In other words, an enzyme that

modifies chromatin. So this, even though it is just a working model, has led us to think that Elongator associates with the elongating form of polymerase as it travels chromatin and enables loosening of the chromatin structure by acetylation of the tails of histones H3 and H4.

As some of you may know, this is an area of very great interest to a lot of people right now, and we have found that mutations in yeast which debilitate Elongator function do, indeed, have a profound effect on the transcribability of genes. It was proposed that we should exchange papers, and will be happy to send interested parties a paper which will appear in next month's Molecular Cell about this issue.

Now, this is a working model. Of course, we need to understand not only what Elongator does in yeast but, more importantly, what Elongator might be doing in mammalian cells. But let me just try to go through what we know from yeast research about the Elongator complex.

We know, as I already told you, it was isolated as a component of the elongating form of RNA polymerase II and the integrity of this holoenzyme I showed you is dependent upon a hyperphosphorylated CTD. So the RNA polymerase II enzyme has a C terminal repeat domain, which is hyperphosphorylated in the elongating form of the enzyme but not in the initiating form. And our complex specifically binds or preferentially binds to the form of polymerase, the elongating form, that has a hyperphosphorylated CTD.

The highly purified Elongator complex that I just showed you binds to highly purified RNA polymerase II. So it is a direct interaction in vitro.

And in vivo, yeast strains lacking all the Elp genes have similar phenotypes. So it is not so that we think at least in yeast that Elp1 does a lot of things and just one of the things it does is with the other Elongator proteins. So we think that the activity, the role of the IKAP protein is tightly coupled to the ability of Elp3 to act as a histone acetyltransferase.

You already heard speculations about where is Elongator. Of course, you need histone acetylation both in the cytoplasm in order to enable assembly of chromatin when the nucleosomes are transferred to the nucleus. But, of course, you also need histone acetylation during transcription because you need to have an open chromatin structure that enables polymerases to travel through chromatin and to associate with chromatin in the first place.

In this respect, it is interesting that yeast strains lacking Elp genes have a number of phenotypes, many of which can be explained by defects in transcription activations, and indeed Elp delete strains show slow activation of some but not all regulated genes. So even though we think that Elongator is quite generally involved with RNA polymerase II, it clearly only affects a small

subset of genes and perhaps transcription of such genes, when we think in mammalian cells, could be important for the development of FD.

Yeast Elp strains are sensitive to the elongation-inhibitor 6-azauracil. So this is a trick that yeast researchers use to try and assess whether certain gene knock-out strains are likely to have a connection to transcriptional elongation. Elongator delete strains are, indeed -- at least some of them -- sensitive to elongation-inhibitor 6-azauracil.

Now, it was already mentioned that we could do genetic studies of Elongator, and actually that has been done by quite a few researchers. It is very important in this connection to mention that Elongator deletes genetically interact with several transcription related factors, such as CTK1 which is a kinase which specifically phosphorylates RNA polymerase II. If you mutate CTK1 and mutate Elp1, you die. Yeast cells cannot survive this. If you mutate one of the nonessential polymerase subunits, RPB9 and Elp1 or the yeast IKAP homologue, you die. If you mutate the FCP1 gene, which encodes the phosphatase that removes phosphorylation from the polymerase during and after transcription, you also die. So these are clear genetic evidences for a role of the Elongator complex and thereby the IKAP homologue Elp1 in some sort of transcriptional regulation or pathway.

Now, it's important, of course, to mention that Elongator does, indeed, have histone acetyltransferase activity in vitro and that the main target is histone H3. I'll show you more direct data on that later.

Elongator binds DNA and RNA in vitro. So purified Elongator complex binds directly to DNA and RNA. At least as a biochemist, this is very strong evidence that it is relevant to look at what Elongator might be doing in the nucleus.

And as mentioned already, Elongator is found both in the nucleus and in the cytoplasm of cells, and this is something that is very important for us to understand. Our angle on this has been the role in the nucleus, but of course this does not mean that the reason why patients get familial dysautonomia cannot be because of a separate role in the cytoplasm. This is just important to keep in mind in that I am bias in what I tell you now because what I'm interested in first and foremost, in terms of my basic research, is of course what the Elongator complex does in connection with transcription.

But of course, my interest in the familial dysautonomia disease kind of broadens my view on what I should study, and indeed we have started a number of studies which will be addressing the potential role of Elongator not only in the nucleus but also in the cytoplasm.

Now, this is just to remind me to tell you how highly conserved the histone acetyltransferase Elp3 is.

This is an alignment of several versions of Elp3 from many of the sequenced genomes that we have by now. Now, you can't read this, and that's not the purpose. The purpose is to show you all the red regions are identical amino acid residues and all the yellow residues are similar. And it is striking that Elp3 protein is more than 75 percent identical at the protein level from the lowly yeast, the single cellular organism of yeast, to man. So this is an unusually conserved histone acetyltransferase.

Now, using antibodies that we got from Claus Scheidereit against the IKAP protein, very, very specific antibodies, which recognize a little C terminal tip of the protein, combined with antibodies against the human Elp3 protein that we raised ourselves, which is also very, very specific, we simply followed Elp3 and IKAP protein during a very long protocol of conventional purification, pouring extracts onto columns and seeing where the proteins come off. They usually always follow each other, but it is important to point out that there are fractions containing IKAP not containing Elp3. We're not sure how important this is because we also know that the Elongator complex, unfortunately, is quite fragile, and as soon as you subject these complexes to purification, you are in

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another situation that you are in in the cell, and we could, therefore, not be sure whether these fractions of IKAP not containing Elp3 are actually relevant at all in vivo or in the cell.

Now, the first thing we purified -- we did this the brute force method. You see many, many columns of purification and hundreds of liters of HeLa cells that we grew to make this possible. We end up with a three subunit complex, IKAP, human Elp2, and human Elp3. So these are all homologues of the yeast proteins, Elp1, Elp2, and Elp3.

Now, we knew from yeast that there had to be more to the story than just Elp1, Elp2, and Elp3, and knowing this, we went back and did a more careful study of the Elongator complex using this IKAP antibody to do a mild purification of Elongator. When we did that, we actually found that just like in yeast holo-Elongator complexes six subunits, IKAP, Elp1, Elp2, and Elp4. And I'll show you that we now know the identity of p38 and p30.

So, Elongator, six subunits in both yeast and man, all are homologues. So this is very striking. It's likely we can learn something from yeast in terms of what human Elongator might be doing.

I should also mention that this human Elongator

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complex was purified from the nucleus. It was a nuclear extract, but also that some recent results that I did not want to bring here because I'm not sure about them yet indicate that perhaps Elongator might be slightly different in the cytoplasm than in the nucleus. So when we do a very short purification procedure, which skips some of these steps, but most importantly retains this

one, we get the six subunit complex from the nucleus, but something quite different and potentially bigger from the cytoplasm. We do not know the significance of this yet, but this is something we are definitely addressing right now.

Now, importantly, this holo-Elongator complex that I showed you, the six subunit complex, but not core Elongator, has histone acetyltransferase activity in vitro. This is just a comparison of the activity of this core complex with that of holo when we add radiolabeled acetyl CoA to a reaction with the holoenzyme and purified histones. We find that core enzyme has no HAT activity whereas the holoenzyme, the six subunit complex, has HAT activity directed against histone H3 and, to a much lesser extent, histone H4.

This on the right here is just a control to show that holo contains the Elp4, 5, and 6 protein, but core does not.

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Now, when we did studies of where we can find Elongator, that is, using the very specific IKAP antibody and our very specific Elp3 antibody, we got a result which is actually surprisingly dissimilar to what others have reported. We found that in our hands the majority, as you can see here, is clearly in the nucleus, whereas others have found it predominantly to be cytoplasmic. But you cannot see from our research that that's clearly a lot of Elongator in our hands in the cytoplasm. It is an important issue for us to understand why do we find Elongator where we find it. Can it change localization with stress signals, as has already been suggested? And what is the role, if any, of Elongator in the cytoplasm versus the nucleus?

So in terms of the human data which have been derived by us and also by a study by Danny Reinberg and his co-workers, human Elongator also binds directly to RNA polymerase II in vitro, something found by both groups. Very nice experiments by Reinberg and co-workers show that HeLa nuclear extracts depleted for human Elongator can transcribe naked DNA in vitro, but seems to be severely impaired for transcription of chromatin templates, a very strong indication in my opinion that there is some connection, a direct biochemical connection, which of course are the connections that I tend to believe the

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most, that Elongator is, indeed, involved in chromatin transcription.

I also want to remind you that it's been shown already by Scheidereit and co-workers and over-expression of the IKAP protein abolishes transcription activation of several genes in human cells, again indicating there's some sort of role in transcription for this protein. As has been pointed out several times also by me now, a significant portion of Elongator is cytoplasmic found by Reinberg and co-workers, and we also see a very significant portion in the cytoplasm. So it's not only Reinberg and co-workers.

And the mouse Elp2 protein StIP1 seems to be translocated to the nucleus upon cell stimulation, which is work by Schindler and co-workers a couple of years ago published in PNAS, which I am sorry to miss out here.

Now, of course, one of the things that we were given a grant to investigate was IKAP interaction proteins. It's already in the database or in publications at least what Elp2, Elp3, and Elp4 are, but also again, by cutting out these bands and sending to them our collaborator Paul Tempst to identify, we now know what these proteins are.

So the identity of the human Elp5 gene is here. This is the gene number that we found. We find virtually

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identical genes on chromosome 16 and 17, or we don't but the Yeast Genome Project, of course, has done this for us. So this is one thing that is so powerful about biochemistry these days, and something that is often overlooked. You think that the genome was for geneticists, but it is actually also truly for biochemists in that as soon as you identify a protein, you immediately know where the gene is. You immediately know where the gene is and whether there's a connection to human disease. And I think that is something that I have not even had time to look at yet, whether there might be some connections between these gene regions and human disease. And I'm sure that lots of people in the audience here are probably better placed to do that than I am.

The Elp6 homologue, again the accession number here, seems to be encoded by a single gene on chromosome 3. One thing I didn't point out is here's the homology indicated to the yeast protein. All the red bars are identical residues. All the yellow bars are similar residues. So the homology of these proteins to the yeast counterpart is visible but not compelling and therefore it was only by doing the mass SPECT analysis that we dare trust that this is, indeed, the homologue. We could not find this by searching the genome with the yeast sequences. We had to get hard core data.

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So the title of my talk, I should say, is not my title because I do not really think I'm in a position to speculate on the specific role of Elongator in the courses and maintenance of FD symptoms. But I can, of course, more broadly speculate about what I think IKAP and Elongator might be doing, and I think it's implicit in what I've been saying already what I think might be going on.

It is, of course, important for us to discover. This is indeed a major part of what we are trying to do now in the human Elongator part of my work, and that is, the question is whether FD is caused by its transcription defects due to impaired Elongator function. One thing we can do is to ask whether functional Elongator complex actually assembles in cells which expresses the truncated IKAP protein. And this is not easy because we rely on cells from patients. So what we are doing right now is to

try and express truncated or FD-mimicked proteins which has an epitope tag. So, we can actually pull out the shorter version of the IKAP protein and see whether it still interacts with Elp2, 3, 4, 5, and 6. And these experiments are still in progress.

Of course, once we have pulled it out, if it is there at all, we can ask the question of whether this complex is functional. Does it bind DNA? Does it have

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histone acetyltransferase activity? Can it transcribe chromatin in vitro? So very, very basic biochemical techniques, which do not necessarily tell us what is the cause of the disease but at least gives us some insight into the function of the IKAP protein.

I should emphasize again that we have failed to pull out IKAP with IKAP-specific antibodies without also pulling out Elp3. So I want to emphasize that the role of IKAP I think is tightly connected to that of the histone acetyltransferase Elp3.

Now, this might prove that I don't know enough about familial dysautonomia, but I was under the impression that some of the patients do not have the classical IKAP mutations. Of course, it would be very important to discover whether such patients, if they exist at all, actually carry mutations in the other Elongator genes. It is actually puzzling that it is only mutations in IKAP that give you these symptoms, this disease.

But I want to remind you that it might be interesting to compare the symptoms in FD patients and patients with transcription-related diseases such as Cockayne's syndrome, which is due to mutation in a protein which is also known to be involved in transcript elongation. One of the important symptoms in Cockayne's syndrome is actually also neurological disorders and the

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patients with Cockayne's syndrome also often suffer an early death. So, again, I'm not a doctor and I can only give you wild speculation, but some of you who know more about symptoms and comparisons of them might want to look into this possibility.

Of course, as I told you, we need to know whether IKAP is also a component of other protein complexes than Elongator. And if so, what is their function and what are the subunits? This is something we're actually pursuing as well.

Finally, I just need to acknowledge the people who actually did the work I presented today. This was mostly the work of a very talented post doc in my lab, Nicola Hawkes, who is right now supported by the Familial Dysautonomia Foundation, that is, the work on the human Elongator complex. And this partly was started by Gabriel Otero when he was a post doc in my lab several years ago.

I should also thank Paul Tempst's lab at Sloan-Kettering who has been doing all the mass SPECT analysis of the proteins we isolate, and Claus Scheidereit in Berlin who has supplied us with a very, very good reagent, the IKAP antibody, which unfortunately, in terms of FD, is

against a C terminus which, of course, does not exist in the disease-causing protein.

And then I should also thank Michael Dahmus who helped us with some of the functional assays on human Elongator.

Thank you very much for your attention.

(Applause.)

DR. AXELROD: Our next speaker is going to address the question about secondary expressions of other neurotransmitters. The title of the talk is secondary neurotransmitter aberrations as demonstrated by immunoreactive stains in dermal neurons, and it will be by Dr. William Kennedy.

DR. KENNEDY: I'd like to express my appreciation to Dr. Axelrod and to the foundation.

I'm going to report on a collaboration between Drs. Hilz, Stemper, Axelrod, and myself in Minnesota with Gwen Crabb, and Gwen did most of the work on this.

I'll first talk about the methods that we used because they may be a little bit new, findings in FD, and then as requested by the committee, an overview, trying to fit this with other disorders and the general use.

The neurologists would call me an electrician. I'm a neurophysiologist, and for many years I have looked at pancreas transplant recipients. And I found that using most of the tests that Dr. Hilz reported upon and following pancreas recipients -- and these are diabetic patients who are cured of their diabetes. It's no longer necessary to take insulin. I found that the patient was better but my tests weren't, and this was very frustrating. So I thought I must start looking at the nerves themselves to see what they look like. This was about 1990.

Fortunately at that time, three things were happening. One, Thompson in England had shown us a protein gene product 9.5 that is present in all central, peripheral, and autonomic nerves. At the same time, the confocal microscope was making its appearance, and I'll talk more about that in a moment. And we were able to, on the same selection of the same nerve, do double or triple staining so we can look at several antigens.

Confocal microscopy is much like the CT of the brain that you know so much about. One can take a thick section of tissue and cut it -- optically section it, not cut it. These are two micron slices. We take 16, 20. We can take 50, and then you can page through these like going through pages of a book and follow nerves and follow their direction and their course. And you can look at each individual one or you can put them all together, and that's what we'll be doing today, looking at their combined so-called Z series.

On each section, fortunately, there have been different fluorfors brought forth, mainly by Jackson Immunonuclear Labs, and by using three individual spectrum

and separating them by filters, you can look at one, two or three, combining each to different antibodies. So you can do so-called triple staining and look at three proteins in the very same nerve fiber in the very same section.

This is just a quick orientation to skin. Not all sections of skin look like this with all these structures. This happens to have sweat glands, hair, a large artery in it, and I'll talk in a moment quickly about the nerves. This is an artery. The nerves here are yellow or green. You see the arteries are almost covered with vasomotor fibers and autonomic fibers. Two sweat glands here. The sweat glands are not stained, but there is so much nerve you can get an idea of the tubules of the sweat gland. In addition to being stained for nerves, this is stained for blood vessels. You'll see all the capillaries. Blood does not have that many capillaries ordinarily unless there are sweat glands in the section or hair follicles. This has both.

Following up, this is a hair follicle. It's not cut so you can see the nerves, but you see the outline of a sebaceous gland, and hanging off the chin is the beard, which is made up of nerve fibers and arrectores pilorum muscle, which gives you goose bumps. We're from here on

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interested only in the epidermis which is up here. It happens to be a diabetic patient so there are only two little nerve fibers here, but we can see nerves in epidermis better in this normal section.

This is the epidermis. This is the dermis. In between, we've stained the basement membrane with collagen IV, so this reddish thing here is a basement membrane. It's a very thin layer. It looks thick there because this is maybe 20 sections and you're looking at more of a profile rather than on the edge.

The same collagen IV is in the basement membrane around blood vessels. Here you can see the blood vessels. Here are the nerves down here, nerve trunks. A nerve trunk here goes up. It innervates sort of a segment of skin. And fortunately for us for quantitation, the nerve trunks branch into single nerve fibers just below the basement membrane. For example, here you see a single nerve fiber going up, another nerve fiber going up. So if they're single, you can count them and therefore quantify rather accurately.

And to give you an idea of the thickness of the section, you see back in here? It's a dermal papillae going up. This capillary will go up and end in a capillary loop, but it's deep in the section so you can't see it clearly. But we could thumb through it or page

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through it and see it very clearly.

Dr. Axelrod and her group sent me tissues from 10 subjects, mean age of 34. She had biopsied the calf and the back. We had normal in our laboratory. We had informed consent and IRB approval to do the work.

Quickly, these were 3 millimeter biopsies, about

an eighth of an inch in diameter, fixed, cryoprotected section, and mounted and examined in the confocal microscope. And this is a program by Michael Breakefield to quantify those nerve fibers.

Here are two sections, normal and from an FD patient. Again, you see the blood vessels and the nerves in the epidermis. We're going to quantify these, but also we're trying to quantify the nerves under the basement membrane which you see here, and it's a nice capillary loop. These are more difficult to quantify.

Normally these do not stain for substance P, calcitonin gene-related peptide, or VIP. The first two substances, P and CGRP, are often seen in this area in normal skin. From an FD patient, you see quite a difference. There's only one nerve fiber in the epidermis as opposed to many. Very thin subepidermal neuroplexus, which I may revert to call SNP.

We quantified in two ways. This is a very simple way, but it's very effective. That is, the way we

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usually quantify anything in ordinary life: we call it normal, which would be 0; mild, moderate, severe, 1, 2, 3; or minus 4, there's nothing there. Absent.

This is the back and the calf. Let's look at the calf first.

These are nerves in the epidermis. You see almost all of them are minus 4. So there were no nerves, very few nerves in the epidermis. Now these nerves are nociceptors for mechanical pain, that is, pin, or for noxious, heat pain such as touching a hot stove, and Dr. Hilz tested for those.

Over here in the back -- and we chose back because it's more proximal and most neuropathies are distal, affect the distal extremities, mainly starting in the feet. So we thought, okay, we'll go very proximal, close to the spinal cord, but we were fooled in that those are affected also.

We did that for diabetes too, and we were fooled there also. They were affected.

And then we quantified in the same method the subepidermal neuroplexus, and you see those are affected rather severely too, not quite as bad as this. But if you take the average, it's almost the same. Severe here too. This would be moderate in our language, but pretty much involved.

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I mentioned CGRP and substance P. Normally we always see those. None, none, none. There's one fiber in all 10 patients.

I mentioned in passing VIP, vasoactive intestinal polypeptide. We never see that normally up in the top of the skin near the epidermis or just under the basement membrane, and we didn't see it in the calf. But here it was present in all the patients.

Now, we've done regeneration patients mainly on mice but also some in humans, and so has Story Landis, and we find this if their nerves are regenerating. So this is

somewhat of a hopeful sign. We're not sure if those nerves belong down where they're usually seen in sweat glands and around blood vessels and are aberrant, are someplace where they're not supposed to be, or if for some reason, the neuron elaborates VIP for a short period of time and we see it. So it may be a sign that there are some efforts for regeneration.

I'll go through some slides of FD patients from showing no nerves to a small innervation down here below the basement membrane. A couple of nerve fibers. A few more here. A little better. A couple fibers up here, but a little more down here. Almost to normal except there's a branch over here where there's not much. And absolutely absent nerve fibers. So that's about the range we saw.

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We also quantified by counting the nerve fibers. The normal in the back is much higher than in the calf, 70 versus 16, and this is per millimeter of epidermis. We can also do it per area, so you can compare one lab to the other. This is easier to present.

In the back, if you use this as the normal, this was the best, less than a third of the normal. The rest ranged down from there.

In the calf, this is normal. You see this is the mean, less than one nerve fiber. So almost absent.

So the sensory tests Dr. Hilz was doing -- this is one explanation why they're abnormal, at least in those two areas. I assume in other areas too, though, we'd find the same.

Another way to look at it, the normal range for the back. By back, I mean T4-5, just off midline and 1 standard deviation on each side. And here are the findings in the patients. This is a range for the normal calf, and the findings in the patients.

Just something about VIP I mentioned. This is one of the patients from the back that had some. If we do look at the VIP, well, that stained different fibers plus those that we saw. And if we look at double staining, you can see these were not stained for VIP. These had both VIP and PGP 9.5. So that's how you can see. We could do

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triple and show another substance in that nerve too.

This is another interesting fact. This is S100 antibody and it stains the Langerhans cells, but also it stains the sheath cells, the Schwann cell sheaths here, here, here, here, here. Now stains that just show the PGP 9.5, which is only in the axon, and we see they're here too. But that nerve fiber does not show the axon. If we do the double staining, well, we can see both here, here, here. But this one just has the sheath. So it's an empty Schwann cell sheath. And we saw this several times. So there's some sign of progression. A Schwann cell sheath will eventually disappear too, but much, much later after the axon.

I put this in here because we saw this curious excess of capillary loops. We're not sure what it means and we didn't follow it up. We're not capillary people,

and we hope to find somebody to help us with this.

So in conclusion, in the FD patient, we saw the epidermal nerve fibers decreased in density. Substance P and CGRP loss was observed. VIP, usually absent, was up near the epidermis. Empty Schwann cell sheaths suggest some progression. Capillary abnormalities were observed. We don't know what they mean. And FD patients, therefore, have profound changes in the sensory innervation of the skin.

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Myself and Gwen Crabb did most of this work and a lot of people in the lab helped us, particularly Dr. Selim, who is a post doc from Cairo.

Now, I was asked by the committee to look in a more overview fashion. What I showed you were unmyelinated nerve fibers, and I told you that we happened in 1990 to be at the right place at the right time when PGP became available, the confocal microscope became available because neurologists, or anybody else, were not able to see unmyelinated nerves very well before. In fact, a very famous Mayo Clinic dermatologist said in 1988 that there's no good evidence that unmyelinated nerves exist in human epidermis. And he was good at staining skin. He was very good. He invented many silver stains. So it wasn't him. It was the methods. So we're very fortunate.

Now, unmyelinated nerves are the majority of nerves in peripheral nerve, and they make up almost the entire innervation to the autonomic nervous system. So suddenly we're able to look at nerves that we couldn't see before. It's like opening up the West to the settlers. There will be a stampede to look at unmyelinated nerves in other structures because that's the majority.

So the start was in skin, and I'll speak just a little bit about skin on these unmyelinated nerve fibers

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that I've been showing you, these nociceptors to epidermis.

Well, they're abnormal or missing in many places where we'd expect them to be. We looked at FD, congenital insensitivity to pain and hidrosis and some others where we would expect them to be abnormal. Here's an entity, patients that show up with burning feet. Neurological is often normal. Sural nerve biopsy is sometimes normal. Being smart, neurologists sent them to psychiatrists. Well, they have severe abnormalities of unmyelinated nerves, so this is a syndrome. It may be the most common type of neuropathy.

We expected unmyelinated nerves in the epidermis to be absent or reduced in diabetes, HIV, and leprosy, and they've been shown to be so.

I'm not so sure we expected them to be reduced in Friedreich's ataxia which involves the spinal cord and the large nerve fibers. Maria Nalano in Napoli showed that they're almost absent in Friedreich's.

Guillian Barre. Well, maybe they should be involved, but I'm not so sure in these.

Here's spinal and bulbar muscular atrophy, which I have a personal interest in. I've only looked at one patient, but we'll look at more with the folks at NIH. They were severely involved. That's a motor disease with some sensory conduction problems. It's not a sensory disease.

Well, are these unmyelinated nerves some surrogate messages of something is wrong with the nervous system? Well, we'll go further. That is, what about other organs? What about the gut?

We started with gut because of my interest in the diabetic patients. Many of them have these same complaints as normal people, but they're more severe. We've been diagnosing those patients with autonomic neuropathy for over 50 years, and it's a certain diagnosis. We know they have it. We've never proven it pathologically. We do it by tests that Dr. Hilz did, sweat tests and other tests, which have never been pathologically proven to have abnormalities.

This is a jejunum stained for nerves in the green and blood vessels in the red. We'll look at one of those. Here it is. And if we just look at the nerves, you see it's loaded with nerve fibers. Here are the blood vessels loaded with capillaries. Crypts down here. We see a nice neural pattern around those crypts. We see many nerve fibers. These are the enterocytes. So this is the surface of the intestine. They're right under the enterocytes, little nerve endings or naked endings. So there's no apparatus there. So it's quite a complex situation.

Here's a mild and a severe from a diabetic and the normal here. These villi are longer. They have longer enterocytes, so there are more enterocytes so the villi grow longer. This particular one looks rather full. We didn't see many of those little endings I showed you. It's maybe a little disorganized here, but I'm not good enough to recognize that often.

But if you go a step further or maybe several steps further to the advanced case, you see the nerves don't reach the end. These villi are clumped together. There are no enterocytes. I don't know if that's for real or not. We don't know if it's due to the handling, the processing, or whether the enterocytes are lost. If they're lost, it's going to be difficult to absorb and secrete from these villi. But the nerves are severely abnormal.

So we have to worry about what does that do to function. What does it to do stomach emptying which gives those symptoms that I showed? About half the diabetics have been shown to have delayed gastric emptying, and they have feelings of fullness, early filling, et cetera. Is that because they're functionally without a sensory supply in the tips here? And I was talking about stomach, so in a moment I'll look at stomach, but I wanted to look at

cross section.

Here's a normal villi with a basement membrane under enterocytes. The green and the yellow are nerves. Many in here. You can see a large central vein and some arterioles. The patients had arterioles, had a central vein. There are no enterocytes, so they're clumped together. They're much larger than this one and no nerves.

This is stomach. Normal, somewhat different because of different morphology, but the nerves go right up to the surface. And here there are fewer nerves and they've died back, so to speak. At least they're absent up here.

So this is a method for early diagnosis. You have to go down into the stomach to get that piece of tissue, though. It may be related to the motility problems. It opens a new field of neurological research certainly. I think maybe, though, the skin nerves, the epidermal nerves may be surrogates. It may be easier just to take a biopsy of skin. In fact, we have another method where we just make a blister and look at the nerves in the skin. You don't even have a scar afterwards.

Well, where do we go next? Gall bladder, pancreas, bladder? These haven't really been looked at. And this is bladder. And we're not going to look at them

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today but just to give you an idea that they can be looked at.

Well, what's the importance of all this to FD? Well, diagnosis would be helpful I guess, but when a treatment does come, how do we know it works? Even if we can prevent the disease, there are a lot of people around that have it, and it's too late to prevent it. We like to know if they get better. So, we need clinical ways, morphological ways to tell if the patient is better after treatment. So maybe a skin biopsy or biopsy of the intestine. Maybe bladder will help.

Thank you.

(Applause.)

DR. AXELROD: Thank you, Dr. Kennedy.

Now we're going to come to our final formal presentation of the morning before we have our open discussion, and this is going to be the construction of a mouse model to study mechanisms of pathogenesis in familial dysautonomia by Dr. Xu.

DR. XU: So I would also like to start by thanking the organizer for the invitation.

I'm relatively new to the FD field. So this meeting will be extremely educational on my part.

In my lab, one focus of my research is constructing mouse models to study various human genetic

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diseases. In the past five or six years, we have constructed a mouse model for ataxia telangiectasia and more recently for MBS. These research experiments have convinced me that a successful mouse model would be critical to study the basis of defects in human genetic

disease. Especially exampled by the ATM-deficient mouse, it has been used by a large number of groups now to study the functions of ATM, as well as the basis of defects in AT.

So, when the pathogenic disease mutation was identified in FD, we decided to move into the research area by constructing a mouse model to study the pathogenesis in this disease, of course, with funding provided by the FD Foundation.

So since construction of a mouse model is a relatively time consuming process, the work is not complete, so here I can only share some of the strategies of how to do the mouse model in this case.

So we first have to clone out the mouse genomic DNA of the IKB gene. Luckily for us, after we mapped and screened the exons of the mouse gene -- and also published by a number of groups -- the mouse IKB gene is highly conserved to humans. Especially, lucky for us, the genomic configuration of the mouse, the IKB gene is very similar. It's completely conserved to humans. Like exon

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19, 20, and 21, their sequence is completely conserved. Especially interesting in these studies is the splicing donor sites for the exon 20 is also conserved.

So after we cloned the genomic piece, we introduced a mutation. The first mutation we introduced into the splicing site exon 20 and now we have also introduced another mutation, the minor haplotype mutation into the exon 19.

The gene construct of making mutations -- what we need to do is basically insert a selection marker. In this case it's PGK mu gene, recessant gene, into the introns, between the exon 20 and 21, which is downstream somewhere here. Also this selection marker is flanked by log P site.

So we transfected the knock-in construct into the ES cells, mouse embryonic stem cells, and _____ combination between the flanking sequence. What we'll do is replace the wild type exon, endogenous wild type exon 20, with the mutant exon containing the mutation in the splice site. Of course, in this case the PGK mu gene is also going to be introduced into the endogenous locus.

We know for a number of studies that PGK mu gene by itself has a suppression effect on the transcription through the locus, integrating. So I will have to get rid of the PGK mu gene. In this case we can breed mice which

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carry these mutations with Cre transgenic mice that expressed a Cre gene in a germline, and eventually we'll delete the log P site, leaving only the PGK mu gene and recombining the log P site in the intron 21.

Of course, we know that log P site by itself doesn't really have any effect in the transcriptional suppression or activation. So it wouldn't affect the expression of the mutant knock-in allele.

So our work now has progressed to this level. The most time consuming stage of the project is to

construct the mice. We already have the mutant ES cells.
So just based on my very limited understanding of FD, so when we get the mouse, we'll be able to address a number of very obvious questions to test whether this mouse indeed can be used as a mouse model for FD disease.
Here, as a number of speakers have mentioned, the splice mutant might affect IKB splicing in a tissue-specific manner. So this can be very easily tested also in the mouse model.
Of course, with the mouse, in collaboration with people here, we can have a very comprehensive analysis of the different tissues for the defects observed in FD.
So I will conclude by thanking the foundation for funding, as well as the two persons in the lab now that are focusing on this project. Thanks.

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(Applause.)
DR. AXELROD: I'd like to thank all my speakers this morning, and now I'd like to invite them all to come up and sit at the table. We'll have to add about three more chairs to this table. I'll invite our moderator, Dr. Rubin to come up and moderate an open discussion. I hope you're all stimulated to ask lots of questions, and we'll try to answer them.
Dr. Rubin?
DR. RUBIN: I guess everybody has settled in. I have the easy job as serving as moderator. I wanted to thank the organizers for making it possible for me to be here, and I wanted to thank FD Hope for supporting the research in our laboratory and for their efforts to ensure my presence here at this meeting today.
If you have questions -- and I'm sure there will be many -- if I could ask if you would move to the microphone so that people can hear the questions throughout the audience, that would be helpful. Go ahead.
DR. HIRSCHHORN: We've had a lot of talks and couldn't ask questions in between, so I've got a few comments and questions. I'm Kurt Hirschhorn, by the way. And they're addressed to three, perhaps four people.
The first is really sort of a suggestion for Sue in terms of the tissue culture question that you're

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asking. What's happening in the tissue culture?
Many years ago, several labs, including ours, showed changes in activity of specific enzymes involved in in-borne errors in the carriers for those, but also to some extent in the affecteds, during stimulation of lymphocytes by PHA. The first of these actually was ours with alpha-glucosidase. The second was by Joe Goldstein with S-methionine synthase, and the third was by my wife Rochelle with adenosine deaminase. I would suggest that you might try to see what happens to try to explain the differences between the various labs in terms of what you consider in the log phase versus stationary phase to see what happens to these resting lymphocytes when you stimulate them with PHA and they go into a log phase. That's the first thing.

The second is a comment about something that was said by Felicia and actually by a couple of other people too who said, well, it's very important to look at the proteins and genes affected by IKAP and perhaps its changes because this could explain a lot of the things.

Well, one of the things that will be going on with an enormously exponential increase over the next few years is the search for modifier genes. There is no such thing as a mendelian disease anymore. They all are going to vary. They're all going to be varied by mutations,

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variations, and so on and a whole host of other genes that are going to affect the expression of the particular gene that we're talking about.

So the search perhaps should not only be for those proteins and those genes that are downstream from IKAP, but much more important perhaps, by things that are upstream from IKAP that might affect the activity of IKAP and even possibly its splicing.

With that in mind, Dr. Svejstrup, I have a question regarding the transcription factors. One of the transcription factors that seems to be necessary in terms of its activity before histone acetylation is the whole SWI-NEF complex which opens chromatin. And that was first, of course, described in yeast. And I'm wondering whether you have any idea of an interaction between this and the Elongator situation.

DR. SVEJSTRUP: We have looked for such evidence both biochemically and genetically, and one thing that should be mentioned about the SWI-NEF complex is that that started out as a nice single model. It has now become so varied that there are so many of these complexes that studying this is a major endeavor. There are so many possibilities. But we are trying, but we have yet to find any genetic or biochemical interactions. We know better now where to look, so I think our studies will be more

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directed in this direction because is obvious to look for interactions between Elongator complex and such other chromatin binding complexes.

We know there's a very strong functional overlap between the Elongator complex and the classical transcription with histone acetyltransferase called GCN5 which is the first one to really be discovered as a histone acetyltransferase that is involved in transcription. If you mutate both GCN5 and Elp3 at the same time, actually just point-mutate them so that they no longer function as HATs, then yeast cells are very, very sick.

The study that's coming out soon and which, as I said, I'll be happy to make available now is showing that these cells have a very severe defect in acetylation of the whole genome, and that this leads to consequences for the transcription of several genes which become hypoacetylated, specifically in the coding region, and are lowly transcribed.

So, I think in terms of all the very nice cell

biology that has been done on FD, it would be interesting to try and investigate acetylation levels in chromatin and perhaps in cytoplasm as well of FD patient cells. They're a very powerful tool for this, and I think time is right to do these kinds of experiments.

DR. HIRSCHHORN: Thank you very much.

Sort of as a last comment, I'm hoping that our moderator will address some of what he might be doing in relation to clarifying the differences between the two original --

DR. RUBIN: Go ahead.

DR. HARDY: John Hardy, National Institute on Aging.

I'd like to ask Dr. Svejstrup if the exon 19 mutation is conserved. Well, of course, the original minor _____ is conserved in yeast, and if there have been any attempts to just putting that mutation into the yeast gene to see what the effects of that mutation are on the complex and so on.

DR. SVEJSTRUP: This is a very good experiment. So, first of all, Saccharomyces do not have very much splicing.

DR. HARDY: Sure, yes.

DR. SVEJSTRUP: So there is no splice donor and acceptor in that region. It's just a contiguous gene.

But we have tried to make the mutation, and I have to say for reasons that are beyond me -- and we're looking into this right now because it was published last week by another group that when they do a C terminal truncation of Elp1, they see phenotypes that are

consistent with the failure of Elongator to function. So this is consistent with a role for the C terminus in normal Elongator functioning, also in yeast.

We did this mutation as soon as heard about the IKAP results, and we have not seen this. But we not have looked at the right phenotype, and it would be interesting if there's one phenotype that is affected by this mutation and others that are not. It would actually be the first time for us to have a separation of function mutation in any of the Elongator genes because no matter which one you knock out, you get exactly the same phenotype. So this would be very important.

DR. HARDY: Isn't the minor mutation a missense mutation? That might be a better one to try in a sense. It's a more selective effect perhaps.

DR. SVEJSTRUP: It's a phosphorylation site mutation, as I remember it, and that phosphorylation site does not seem, as I recall it again, to be conserved in yeast.

DR. KALLUNKI: Hi. My name is Tuula Kallunki and I'm the senior author of this paper that Dr. Sonenshein mentioned in her talk. First of all, I would like to answer her question, and then I have a couple of questions for you.

She wanted to know why there was not a total

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overlap between JNK and IKAP in this immunocytochemical picture that we showed in the paper. And my answer for this is that we had a technical problem there because the antibody that is normally used to detect JNK in immunocytochemistry does not work under conditions that the IKAP antibody works. So then by post doc Christian tested another antibody which doesn't give as nice a staining. But if you look at the papers where people show JNK staining with this antibody that is normally used, it's much more similar to IKAP staining.

I have one question for Dr. Slaugenhaupt. I would be interested to know, because it wasn't clear to me from this talk, what about the carriers? Do they also express mutated IKAP or not?

DR. SLAUGENHAUPT: Yes, they do. When we look at the message levels, we can see a very faint band of the mutated, as you would expect.

DR. KALLUNKI: Okay, but there's absolutely no phenotype. Right?

DR. SLAUGENHAUPT: As far as I know from discussing with the clinicians. That might be better directed at Felicia to address phenotype in carriers, but as far as I know right now, there's none.

DR. AXELROD: The carriers manifest no sign of the phenotype at all.

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DR. KALLUNKI: Okay. Do you think it's kind of a dose effect?

DR. SLAUGENHAUPT: Exactly, a threshold effect is our guess.

DR. KALLUNKI: I also have a question for Dr. Svejstrup. My first question for him actually was about reconstitution of the holo-Elongator with this mutant IKAP, but that was part of the question of the previous person.

But then also I would like to know if you have any experiments underway or if you're planning to do anything on studying the possible role of IKAP in stress response.

DR. SVEJSTRUP: First of all, I should say I have only one post doc working on this issue in human cells. We do other studies in human cells, most notably on the Cockayne's syndrome. That's why I mentioned this as a possible interesting correlative. But most of our work is in yeast, and we simply do not have hands enough to do all the things we'd like to do.

I am actually trying to recruit post doc students. If any of you here are people who are interested in studying FD, please send them my way if they want to do biochemistry.

We have no plans of studying stress responses

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because we do not have the right tools to do so. We will focus on purely biochemical experiments and try to figure out what Elongator complexes from IKAP patients, albeit artificial IKAP patients, if you know what I mean, by

expressing mutated versions of IKAP, how they behave in our biochemical assays basically.

I should comment because you asked about the integrity of the Elongator complex in IKAP patients. We don't know about in the patients or even in mammalian cells, but we do know that if we express the truncated Elp1 protein that mimics the IKAP mutation, we still have Elongator complex. So it doesn't seem to completely fall apart, but this, of course, does not argue that it is not unstable and therefore useless. But we can in IP, immunoprecipitation, pull down Elp3 and we can detect this truncated message. So it is not in itself inherently unstable, it seems. But much more work needs to be done before we understand.

DR. KALLUNKI: Thank you very much.

DR. MAAYAN: Channa Maayan from Hadassah, Jerusalem. I have two comments and one question.

One comment is for Dr. Kennedy. We have just concluded the study on the innervation of the gastrointestinal tract, especially the small intestine, and we also saw an increase of density of the innervation

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and especially the NOS activity.

Another comment is for Dr. Hilz. Among other factors that affect instability during sleep is definitely the decreased lung volume that they have because of scoliosis. This definitely will increase the instability during sleep.

The other question is also for Dr. Hilz. Many years ago, we saw an increase of the parasympathetic heart rate variability, especially in the high frequency band, when they went from supine to standing. You showed that there is a decrease of the baroreceptor especially in the high frequency band. How do you explain the difference?

DR. HILZ: Did I correctly understand you that you said you saw an increase of the parasympathetic during standing up? I think I'm aware of that paper on the baroreflex function.

I don't have a good explanation for that because when we did selective -- I mean, we analyzed, as I showed in one of the slides, the heart rate variability in the supine and standing positions and we didn't see any major change. However, when we did selective stimulation with the sinusoidal stimulation where you can really separate sympathetic and parasympathetic responses, again we didn't see a response.

Perhaps there are differences between individual

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patients. Dr. Axelrod always pointed out that there is quite some heterogeneity in the clinic in manifestations. So that would be my own explanation.

From those two studies, we did not see an increase of parasympathetic response, not even in the sense that when you look at the cardiovagal balance and the comparison of low and high frequency activities, that there is enhanced parasympathetic activity because there is a withdrawal of sympathetic response. So I have no

clear cut answer.
And the other comment. Of course, reduction of lung volume due to the orthopedic problems just adds to the risk of the patients, particularly at night. So I fully agree with that.
DR. HARDY: John Hardy, NIA, again.
My apologies. This might have been dealt with. I missed the first hour. But is my understanding correct that there has just been found two mutations so far in this disease, or have others been found as well? And if only two have been found, have other populations been looked at for the occurrence of FD?
DR. RUBIN: Dr. Slaugenhaupt, would you take that question?
DR. SLAUGENHAUPT: To date we've only identified two mutations in the Ashkenazi Jewish population. Clearly

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there have been, for many years, reports in the literature of patients with FD that don't have Jewish ancestry and other FD-like syndromes. Jesper actually touched on this. We are starting to look at these patients to see if they might have other IKAP mutations, and there's also the possibility that these related disorders may be due to mutations in the other Elongator complex members. So only two mutations and we don't really know the answer to the second question yet.
DR. RUBIN: Let me just follow up on that. I seem to recall the paper that was published by yourself and others that reflected on a non-Jewish person who had -- I'm sorry -- a child born to a Jewish mother or Jewish father. Only one was Jewish and the child had one of the mutations. Can you elaborate on that?
DR. SLAUGENHAUPT: That was a haplotype paper that we published a couple years ago, and in fact, there is a patient that is heterozygous for the major FD mutation and we have not yet identified the mutation, if there is a mutation, on the other strand in that patient.
DR. RUBIN: So there may very well be other mutations in other populations.
DR. HARDY: Thank you.
DR. RUBIN: Go ahead.
DR. GROSS: Hi. My name is Bob Gross. I have a

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question for Dr. Svejstrup.
On your slides with the staining for the IKAP, you showed a lot of cytoplasmic staining in sort of a punctate pattern. Have you looked in the electron microscope or other ways to see what that is associated with?
And then a question for everybody I guess is, if there is a lot of cytoplasmic localization, has anybody thought of a function or begun to look at a potential function for IKAP as part of some other complex perhaps in the cytoplasm?
DR. SVEJSTRUP: As I mentioned, in terms of cytoplasmic function, what we can do is to try to isolate these proteins and try to understand where they interact.

We have, as of just last week, identified with these very powerful anti-IKAP antibodies the six subunit complex, and we started from the nucleus. But something that seems to be much more complicated and much more subunits is from the cytoplasm. So that's our way of addressing this.

One obvious thing to look for with an antibody is with a JNK kinase, for example, comes down with these immunoprecipitates even though they are much more than that because they're actually highly purified when we get to this point.

So what I have learned from doing it this way is

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that very often we get very good clues to function by finding the proteins because if we find a JNK kinase, we find kinase in a signaling cascade, we are in the direction of, yes, that is what is wrong perhaps if it is in the cytoplasm, the IKAP phenotypes originate.

But, of course, my own bias here is that I didn't start studying this because of familial dysautonomia. I want to contribute to solving what is going on, but our own bias is on things that go on in the nucleus. Of course, for us it is important to show that indeed what Elongator is doing in the nucleus is relevant, and that has been a great deal of our emphasis. Again, a lack of hands is a problem in many labs, and not least in mine.

DR. HUANG: Just a quick comment about the phenotype in both human and the anticipation of the phenotype in mouse.

Oh, by the way, I'm Eric Huang from UC-San Francisco. First, I want to thank the foundation for funding future research analyzing the mutation.

But I want to point out the striking similarity in the neurological deficit in humans compared with a mouse with various neurotropin knock-outs and transcription knock-out where you see almost more than 80 percent of neuronal loss in the sensory nervous system, as

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well as the sympathetic nervous system.

So, for example, the congenital insensitivity to pain in humans has been shown to be a mutation in trkA. So that kind of supports a survival pathway. In the absence of trkA, there's severe cell loss during embryogenesis.

Looking forward, in terms of analyzing the IKAP mutant, I anticipate that this will be a severe mutation which will -- in the mouse mutation, it will lead to embryonic _____. And so that calls for the analysis using a conditional approach.

Also kind of to bring on the perspective in looking for therapeutic means, because looking at these mouse mutant neurotropin mutation where a gene has been knocked out, it's very clear that the neuronal loss happened really early during embryogenesis. So in terms of therapeutics in adult or in postnatal life, perhaps the best way is to look for regeneration rather than therapeutics because there's no neuron to rescue anyway.

That's just my personal perspective.

DR. AXELROD: I think you bring up a very good point in terms of the neuronal loss being early in utero, and we've shown that even at birth these children obviously have terrible problems.

But I think if we also try to avoid the

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progression or the progressive loss, if we can enhance survival of what they have at birth -- because we really do very well with the patients until about the teen years, the adolescent years. We improve their tone and we give them therapy. We can compensate. But then no matter what we do, it seems that in the adult years that progression takes place. So if we can prevent that, that would be wonderful.

DR. RUBIN: Go ahead.

DR. LACHTER: Jesse Lachter from Haifa, Israel. I'm a gastroenterologist.

The first question is for Dr. Kennedy about progression which was very interesting, and you showed an empty Schwann cell. But as Dr. Axelrod mentioned, maybe things are present very early on. Was there a simple progression of the age of the patients? You had relatively older patients. I think you had a mean of about age 34. But you had a span there. As the patients got older, did you see something?

And the second short question for you is do you need only fresh samples or can you take cell blocks?

And my question for Dr. Slaugenhaupt was that various tissues were tested for the mutant versus wild type, but I think you didn't mention GI tissues among many of the different samples which you took from different

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parts of the body. And I would be very interested in finding out if that was done.

And last, I would just mention as a comment, because this is such a great meeting, that some kind of focus groups or break-out groups for clinicians at the next meeting -- I hope it will be only a year from now. Maybe it will be two years from now that the NIH can put together a group like this so there could be focus groups for clinicians in the various areas. We had one a couple of years ago at an AGA and different things like that, but putting these minds together would be very helpful that way too.

DR. RUBIN: Thank you.

Dr. Kennedy.

DR. KENNEDY: I can't tell you today which patients showed the empty Schwann cell sheaths, so I can't tell you the ages. We could easily do that by sorting through. I think it's a little stretched to say that that proves that there's progression. I don't know if it does.

As far as your second question, a month ago I would have said no, but the answer today is yes. One of the pathology residents has had the same question about Hirschsprung's disease, and she said, I don't have any fresh patients, but I have a lot of tissue banked away.

So we've been able to deparaffinize and react with protein
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gene product 9.5 and other antibodies and shown beautiful staining. So suddenly all the tissue banked in all the hospitals of the world may become available, and that's kind of an exciting development.

DR. RUBIN: Sue, did you want to answer that other question?

DR. SLAUGENHAUPT: Yes. I don't remember off the top of my head all the tissues that I had on the slide. I know we've looked at esophagus. I don't know that we got a sample of the stomach or not.

But there's one thing I want to point out about that study and that is that certainly the FD tissue is very scarce and all of those samples came from FD postmortem tissues that we obtained from either NYU or the Brain Bank in Miami and, therefore, certainly don't represent I think what's going on necessarily. We have not surveyed many different sections from different patients to see what's going on.

So although I think that our conclusions that clearly splicing is worse in neuronal tissue are valid. Whether there's more in the heart or less in the heart, exactly looking at what's going on, we would have to look at many different heart samples, for example, from different patients before we can conclusively say exactly what's going on in the different tissues.

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DR. LACHTER: There is a bank of GI tissue, yes.

DR. SLAUGENHAUPT: FD?

DR. LACHTER: All those patients were scoped and biopsied and then operated.

DR. SLAUGENHAUPT: So that's something we could definitely look at then. Thank you.

DR. RUBIN: Go ahead.

DR. PELTZER: Hello. I'm Sonia Peltzer. I'm President of FD Hope, a parent of two children with FD.

Actually I first want to make a comment, and that's with regard to whether or not carrier status does impose any symptomatology. Whereas I clearly think that the phenotype of a carrier is vastly different than the phenotype of an affected individual, I think the verdict is still out whether or not there is some evidence of disease.

Dr. Maayan had written a paper several years ago about the incidence of varicella and that because of the decreased number of neurons, you have decreased risk for varicella infection in affected individuals. There was a very casual survey done of approximately 40 carriers also several years ago. It was never published, did not do any matched control comparisons. But there seems to be a decreased risk for varicella infection among carriers as well or perhaps delayed onset of infection. You would

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expect it in younger years, and these individuals were either adults or much later in life. So it would follow that perhaps doing the same skin biopsies that Dr. Kennedy

has done on carriers might be able to provide that answer for you to see if there is some evidence of disease in carriers.

My question is actually for Dr. Axelrod. I've heard the statistics. Let me first preface this by saying we all are aware you can interpret statistics in different ways. I've heard the statistic that 50 percent of patients will reach age 40, and I'd like to get an understanding of where that number is coming from.

DR. AXELROD: That's the result of a combined study that Dr. Channa Maayan and I did based on data from the Dysautonomia Center because we combine our data. It's going to be coming out in this month's Journal of Pediatrics.

DR. PELTZER: How did that statistic derive? Did you look at a cohort that was born 50 years ago and determine that 50 have survived to age 40?

DR. AXELROD: These are probability of survival. It was done with a group of statisticians and based on births, deaths. It was subjected to statistical analysis.

DR. PELTZER: We've done a little bit more of a different interpretation of probably the same data.

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Again, with stats, you can look at things very differently. But looking at a sample of approximately a third of the patients, which we've had access to, and their deaths over a period of three years, it seems that 50 percent do not survive to 30 if you take the different age groups and add them up. The bottom line I think we can all share is that this is a devastating disease.

DR. AXELROD: Yes, but I think one also has to be very careful how one obtains data and that there is no bias in obtaining the data and that the number of individuals that you're talking about, when you look at data from 580 patients, and if you looked at 30 patients or 40 patients in the last three years, the last three years are not the history of --

DR. PELTZER: We looked at 150.

DR. AXELROD: Well, I'm not going to --

DR. PELTZER: Right. I'm saying I think we have that agreement. I'll be interested in seeing how that statistic was derived. Thank you.

DR. RUBIN: Actually I had that question on my list because there were 580 patients. 60 percent are surviving according to the report that you put up there today. 50 percent of them -- and I don't know how to interpret it as well -- reached the age of 40 or I guess have the potential. Can you just tell us how many

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patients there are of the 580 that are over 40?

DR. AXELROD: I can't give you the exact number, but the present statistic was -- I had said 35 percent were greater than 20 years of age at the present time. I can't give you the actual number at this time. You can look at the paper. The paper will be out in the Journal of Pediatrics this month.

DR. GUSELLA: Jim Gusella, Mass General.

I'd like to go back to biochemistry and ask Jesper a more general question which relates somewhat to what Kurt was asking, and that is, to what degree have histone acetylases been looked at to see what targets they have in the cytoplasm and whether they have specific acetylation targets or not.

DR. SVEJSTRUP: This is actually a surprisingly under-developed area. We do know some cytoplasmic HATs, and the definition is basically biochemical. A cytoplasmic HAT is a histone acetyltransferase that will hit core histones but not histones packed into DNA. And our HAT will hit histones not packed into DNA and histones packed into DNA. So per definition, we are working with a type B HAT because we can do both, whereas the typical cytoplasmic HATs will only hit free histones.

So we don't know very much about histone acetylation in the cytoplasm except that we know that

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histone proteins are assembled with chromatin assembly factors in the cytoplasm and can be purified as such as a component of chromatin assembly factors. And those histones are always acetylated on certain residues. So we do know quite a bit about the positions of acetylation which are important for assembling chromatin. But in my opinion, there's nothing wrong with being a HAT in the cytoplasm and being a HAT in the nucleus at the same time and play a role in both things.

What surprises me about Elongator is that if it played an important role in being a HAT in the cytoplasm, you would expect it to have genetic phenotypes when you combine it with a mutation, when you remove their other cytoplasmic HATs, and then you will be relying much more on Elongator. Yet, we do not have synthetic phenotypes. We do not have a more activated phenotype of an Elp3 HAT 1W which is the cytoplasmic HAT. Whereas, as soon as we start combining with the nuclear HATs, we get a very severe phenotype, indicating the important function of Elongator is in the nucleus so that when you remove two, you have a problem. But when we remove just one HAT, you don't. But again, these things are not absolute.

DR. GUSELLA: And were the experiments that were done to show that the histone acetylases worked on the histone present in the cytoplasm done in such a way that

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they excluded the idea that these proteins could acetylate non-histone proteins?

DR. SVEJSTRUP: Not at all, and many of these histone acetyltransferases have many targets. So we have genetic and biochemical evidence that Elongator, as one of its targets, has histones, but it could well hit other things as well.

DR. BROWNSTEIN: Mike Brownstein, NIH.

Felicia, I have a question for you about samples that have been collected and studied over the years. Has anyone done an extensive survey of CNS neuropathology in the brain samples of these patients? Because if the splicing is abnormal throughout the nervous system, I

guess you'd expect to see more neuronal loss than just neurons in sensory and sympathetic ganglia.

DR. AXELROD: Dr. Zagzag, I'm going to allow you to answer that. Okay? I know that when Dr. Pearson was here -- he left a while ago and he was having difficulty approaching the brain just because of the mass and the technology not available at that time.

DR. ZAGZAG: The answer to your question is Dr. Pearson primarily focused on the peripheral nervous system, and to the best of my knowledge, the neuropathology for examination of the central nervous system is basically undone.

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DR. RUBIN: So my task was to facilitate questions, and I had a whole list of questions here to, hopefully, get the ball rolling, but it seems that many of the questions have been asked by others. I'm just going to ask one or two questions if I might, and I'd like to address the first question to Dr. Slaugenhaupt.

I was just curious. This screening panel of compounds sounds like a very interesting piece of work, but you didn't tell us, I don't think, about the mechanism by which you're evaluating the ratio between wild type and mutant.

DR. SLAUGENHAUPT: For the first-pass screening data, what we did was use the densitometric assay. So we took an FD cell line that was split into parallel cultures and we did a first-pass screen by treating the cultures with the drug and then isolating RNA and evaluating a ratio using densitometric data.

So we take the most active compounds and as we're going back and doing the follow-up, we'll do the follow-up in multiple cell lines and then assay by quantitative PCR for the wild type mutant separately to see if we see an effect.

DR. RUBIN: And did I understand correctly that this is going to be published soon?

DR. SLAUGENHAUPT: The meeting report is up

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there. Our data is not ready to be published on the specific compounds and what's going on with those compounds. We're doing the follow-up studies now. So hopefully we'll have some positive results in which will be published then.

DR. RUBIN: Dr. Sonenshein, we haven't picked on you yet, so it's your turn. Treatment with increasing concentrations of TNF is having some impact on IKAP. Can you tell us is that at the transcriptional level?

DR. SONENSHEIN: We only looked at doing immunohistochemistry, so we have no idea at what level it's affected.

DR. RUBIN: I guess there are no other questions. Go ahead.

MS. DORNBERG: I'm Greta Dornberg, a parent of a child with FD. This is more of a clinical question. There's an FDnet with a lot of people writing about their symptoms, and a lot of the parents are reporting that once

their child is put on IV solution, their child is doing so well and they're not showing symptoms of crisis when awakening. Do you think there's something to that, giving the solution IV rather than the way the children are normally? Is there something in their stomach that's not working correctly, and is that something to do with the hyponatremia we've been seeing and so forth?

DR. AXELROD: Are you talking about IV hydration fluid or are you talking about IV medication?

MS. DORNBERG: Hydration fluid. They've all been writing that even though they get adequate liquids normally.

DR. AXELROD: Yes. Well, sometimes the stomach is not able to absorb the fluid well when they're in crisis, and so the hydration is restoring the vascular volume and the patient is perfusing their tissues better. So they do feel better.

MS. DORNBERG: Is that something, though, to do with Dr. Kennedy's work? Is there something in the stomach that's not really picking things up, whereas it is through the veins?

DR. KENNEDY: We haven't looked, so I can't answer that. But from looking at the peripheral nervous system, those C fibers, I would put a bet and maybe give odds that they'll find abnormalities in the stomach too.

DR. AXELROD: We have some preliminary data that indicates that during crisis that there is a vasoconstriction or poor perfusion of the gut so that even if you're delivering fluid, therefore the fluid can't get absorbed from the GI tract. It's what we would call a vascular problem.

DR. SONENSHEIN: Can I mention that if people do have manuscripts that are in press or they're saying you can get it, if people could e-mail them to me. Then anybody who puts their name on that e-mail list, I will forward all the papers to them.

DR. RUBIN: Gail, I wanted to thank you for that offer and that is helpful.

I did have a question for Dr. Kennedy about the innervation, and I was wondering whether the differences that you're seeing are related to severity of disease or age of the patients.

DR. KENNEDY: Our controls covered a very wide range and even people in their 60s have much greater innervation. So it's not due to age.

Severity of disease I can't really answer because the samples we got -- they were all quite severe, as you saw, absent nerves in many. So we would have to either sample people who were less involved or perhaps different locations would show that.

DR. AXELROD: The other thing is that when sampling all of the patients -- there were only 10. Again, numbers are very important. And the 10 patients were all post-adolescence. So maybe the way to have looked at it was to do biopsies on pre-adolescents versus

adolescents so that you actually could pick up some difference.

DR. RUBIN: Dr. Maayan will get the last question.

DR. MAAYAN: It's just a comment on your question. We were looking at the same thing on the innervation of the gastrointestinal biopsies, and looking at the age and severity. And we were looking at about, I think it was, more than 20 patients. Because the range was not so big and, after all, 20 was not enough, it was not significant. But there were some trends, definitely.

DR. RUBIN: Thank you. I'll turn it over to Dr. Gwinn-Hardy.

DR. GWINN-HARDY: Thank you to all the speakers and discussants in this section, and thank you, Dr. Rubin, for leading the discussion. I can see we have a lot more to talk about, but I want you guys to get some food in your stomachs too. Let's meet back here at 1:15.

(Whereupon, at 12:15 p.m., the meeting was recessed, to reconvene at 1:15 p.m., this same day.)

AFTERNOON SESSION

(1:26 p.m.)

DR. GWINN-HARDY: It's my pleasure to introduce David Goldstein, who is a friend, as well as a colleague. He's the chair of the next session, and he is going to talk to us about dysautonomia in Parkinson's disease as well as help us with the rest of the session and lead the discussion at the end of the session. Thank you very much, Dr. Goldstein.

DR. GOLDSTEIN: Thanks, Katrina.

Well, I think the contents and flow of this session are going to differ drastically from the contents and flow of the first. The first session was highly focused on a specific disease entity, familial dysautonomia, which is, as everybody now knows, a quite rare condition. I'm going to be heading a session that hopefully will expand your perspective a little bit both in terms of other forms of inherited dysautonomia besides FD and also in terms of other -- how do I put it -- other constituencies, including a patient support group and the point of view of the people who are actually severely affected by being in a family with an inherited dysautonomia.

Now, one of the themes in my talk, which I'll be starting in a minute, is that although FD is quite rare,

inherited dysautonomia is not rare. Inherited dysautonomia is actually quite common. This is my

editorial, but probably the most common form of inherited dysautonomia is in a disease where it hasn't thought to be a dysautonomia at all, and that's Parkinson's disease. So, we're going to be shifting from a rare condition that's definitely Office of Rare Diseases material to a common disease that's bread and butter NINDS material, and I think we'll be able to come up with some interesting comparisons and contrasts in the discussion.

The first order of business is to dispel the notion that there's an autonomic nervous system. There are actually five components to the autonomic nervous system. They could be affected or not affected in a particular disease, and I think it's a terrible mistake to lump all forms of autonomic failure together.

Just to refresh your memory, about a century ago Langley defined the autonomic nervous system in terms of three components: parasympathetic, which is a term that he coined -- he also coined the term "autonomic nervous system" for that matter -- sympathetic, and enteric.

In the early 20th century, Walter Cannon, a great American physiologist, added another component which was the adrenal medulla. And he lumped together the sympathetic nervous system and adrenomedullary hormonal

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system, as shown here, into something that's been called the sympathoadrenal system, or sympathicoadrenal system. He was wrong, but this is still a dominant thought in physiology.

Finally, a fifth component of the autonomic nervous system, discussed briefly this morning, is sympathetic cholinergic function where the neurotransmitter is not norepinephrine, but is acetylcholine, and that is the transmitter that's responsible for thermoregulatory, gustatory, and emotional sweating.

So there are five components of the autonomic nervous system, and I think all bets are off at this point as to which exactly are deranged in FD or any other form of inherited dysautonomia because of the view that all autonomic failure can be lumped.

Now, just as there are different components of the autonomic nervous system, there are also different symptoms and signs of failure of the autonomic nervous system, depending on the component that's affected. Here you see the symptoms and signs that would be associated with sympathetic noradrenergic failure, parasympathetic cholinergic, and sympathetic cholinergic. The enteric system remains a complete mystery, or just about a complete mystery, as far as I know, and the

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adrenomedullary hormonal system is rarely studied.

What I'm going to be focusing on today is sympathetic noradrenergic failure which is manifested by orthostatic or postprandial hypotension. And conversely, because the sympathetic nervous system is absolutely required to tolerate simply standing up, orthostatic hypotension is a cardinal manifestation of sympathetic

noradrenergic failure.

Well, orthostatic hypotension occurs remarkably commonly in Parkinson's disease. You just have to measure the blood pressure lying down and standing up, something that neurologists have not, until recently, routinely done. But if you define orthostatic hypotension as a fall in systolic pressure of 20 millimeters of mercury or more between lying down and standing up, then a substantial minority, if not about half of all patients with Parkinson's disease, have orthostatic hypotension, and you can argue about the percents, but you can't argue about the importance I think of orthostatic intolerance, orthostatic hypotension, as an imminently treatable cause of morbidity and mortality in patients with Parkinson's disease.

Now, if a patient has orthostatic hypotension, that doesn't necessarily mean that the orthostatic hypotension is from failure of the sympathetic nervous

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system. After all, just being bed-ridden or dehydrated will cause orthostatic hypotension.

How do you know that there's a problem with the sympathetic nervous system or with regulation of sympathetically mediated vasoconstriction in a response to a fall in venous return to the heart? One way is by looking at the beat-to-beat blood pressure responses to the Valsalva maneuver where the person blows against a resistance for several seconds and then relaxes.

During phase II of the maneuver, as shown on the left, the blood pressure measured beat to beat goes down because of the fall in stroke volume, but then it starts to creep up, and that is because of reflexive sympathetically mediated vasoconstriction. You can think of it simply in terms of an analogy to a garden hose. And you're measuring the pressure in a garden hose, and you turn down the faucet. The pressure goes down. But if you tighten the nozzle, then the pressure can creep back up in that hose, even though the faucet is still turned down. These people normally tighten the nozzle.

Now, in phase IV, after the person relaxes, the blood comes into the heart. The heart pumps the blood into a reflexively constricted vasculature. It's like turning the faucet back on, but you forgot to loosen the nozzle. Because of that, the blood pressure overshoots,

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and that's what you see here.

Now, in patients who have neurogenic orthostatic hypotension, as I call it, during phase II, the blood pressure just goes down, down, down, down, down. That's because the person can't tighten the nozzle. In phase IV, the blood pressure slowly comes up to baseline but doesn't overshoot for the same reason.

So the two manifestations that I use for determining whether orthostatic hypotension is really neurogenic is what happens to the blood pressure in phase IIL and what happens to blood pressure in phase IV. I only define an abnormality if I see both because there are

cases where you can have one or the other, but I'm talking about both.

Now, in chronic autonomic failure, the syndromes have been divided up into three: pure autonomic failure, this large box called multiple system atrophy; and there are parkinsonian, cerebellar, and mixed forms; and then Parkinson's disease with autonomic failure. And I think you can see immediately there's a problem. How do you tell the parkinsonian form of MSA from the autonomic failure in Parkinson's disease? After all, both groups have "autonomic failure." And again, I'm lumping together all autonomic failure as if it was all one thing. And of course, they have parkinsonism. Up until very recently,

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this has been a big problem because although it's thought that a good response to Sinemet would tend to make the person in this category, there are patients with the parkinsonian form of MSA who improve on Sinemet.

Moreover, it's deeply ingrained in neurological lore that if you treat a patient with Parkinson's disease who has orthostatic hypotension with Sinemet, this will worsen the orthostatic hypotension. Therefore, there are many patients I think who don't have the benefit of a trial with Sinemet because they have orthostatic hypotension in the setting of their Parkinson's disease, and this is unfortunate.

By the way, Sinemet doesn't cause orthostatic hypotension.

Now, in Parkinson's disease, where is the lesion? This is the game neurologists love to play. Is it a free ganglionic lesion such as in multiple system atrophy? Well, everybody knows there's a brain problem in Parkinson's disease. If you'll excuse the pun, that's a no-brainer.

(Laughter.)

DR. GOLDSTEIN: And the sympathetic innervation, such as of the heart, should be intact because the problem is with regulation of the nerve traffic to the terminals, but the terminals are there.

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In pure autonomic failure, it's pretty clear, the problem is postganglionic. There's a loss of the nerve terminals themselves, and at least until recently, there was not really well-accepted evidence for central neurodegeneration in PAF.

Well, what about Parkinson's? In order to distinguish these two conditions, we applied fluorodopamine PET scanning. Fluorodopamine is a sympathetic imaging agent that our group developed at the NIH about a decade ago now. A fluorodopamine scan of the heart -- for those of you who are neurologists, this is a heart, and this is the left ventricular septum. This is the free wall and this is the left ventricular chamber. This is where the blood comes out and goes to the brain. And you see a beautiful image of the left ventricular myocardium. It looks like a horseshoe or a McDonald's M.

We applied this in patients with different forms

of autonomic failure. To control for perfusion, we used 13N-ammonium perfusion scanning. Basically all these patients have normal perfusion.

Here you see that in pure autonomic failure, the perfusion is okay, but the heart is gone.

In multiple system atrophy with sympathetic failure, the perfusion is okay. The nerves are there.

And in Parkinson's disease, at least in this

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patient, the perfusion is there. The nerves are gone. Well, that was a weird one. That says that this is a postganglionic lesion? That means that Parkinson's is not just a brain disease?

We had to follow up on that, so we did. And this shows the results of a lot of PET scans now we've done in several groups. All patients with Parkinson's disease and orthostatic hypotension -- every one that we've studied -- has had a marked decrease in fluorodopamine-derived radioactivity and therefore, I believe, a loss of sympathetic innervation of the heart. And there's no overlap between this group and this group at all. This is 2 standard deviations below the normal mean.

The white circles represent patients with familial Parkinson's disease. Now, when I'm talking about familial Parkinson's disease, the way I'm defining it is the patient, as well as at least two other family members have or had Parkinson's disease. So it's a rather conservative definition.

But even by that definition, we were able to find 7 patients. You can see that the majority of them have orthostatic hypotension that's associated with sympathetic denervation of the heart. You can see there was 1 patient who didn't have orthostatic hypotension but

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did have denervation of the heart, and there are 2 patients who did not have orthostatic hypotension and had radioactivity that was within the normal range. You could argue about whether this group is different from that group or not.

I wanted to compare these groups of patients with familial Parkinson's disease who did or did not have orthostatic hypotension. Here's what I found. In the patients with orthostatic hypotension, the plasma norepinephrine level during supine rest was lower. The fractional increase in plasma norepinephrine during standing was decreased. Normally plasma norepinephrine levels double within 5 minutes of standing. This is backwards. Of course, the patients who had orthostatic hypotension had orthostatic hypotension.

Finally, baroreflex-cardiovagal gain, which is to say the amount of change in heart rate that occurs reflexively for a given change in blood pressure, was markedly decreased in the patients with orthostatic hypotension.

Among patients who did not have orthostatic hypotension -- now we're talking sporadic or familial --

about half have a complete loss of sympathetic innervation of the heart. The remaining half have a partial loss of sympathetic innervation of the heart, and a very small

minority have normal innervation of the heart. This means that sympathetic denervation in Parkinson's is characteristic of the disease.

Well, what happens to these patients with partial denervation? This is a patient who happens to have familial Parkinson's disease who did not have orthostatic hypotension and was studied twice. You can see -- it's kind of obvious -- that there's a progression of the loss of the innervation of the heart in this patient.

Here's a different patient with familial Parkinson's disease who we studied after about three years. We're looking at the blood pressure and pulse rate responses to get a measure of baroreflex-cardiovagal gain. But I don't think we have to give numbers here. You can see that at this time the Valsalva maneuver -- well, let's see. The blood pressure goes up at the end of phase II. There's a nice overshoot at the end of phase IV. That is -- anyone?

VOICE: Normal.

DR. GOLDSTEIN: Normal. And here you see the reflexive tachycardia. So the baroreflex gain is excellent.

Here's the same patient but about three years later. Well, the blood pressure goes down a lot. You can

make an argument here, well, maybe there's some return up here. There is still an overshoot. This is still normal by my criterion. But you can see that the heart rate response is obviously blunted even though the fall in blood pressure is greater. You can even make a case for autonomic bradykinesia in this patient because the heart rate increase is late. But the bottom line is that the baroreflex gain has gone down tremendously in this individual with familial Parkinson's disease.

We've had 2 patients with familial Parkinson's disease who we followed over three years. Obviously, this is preliminary and hasn't been published. The left ventricular radioactivity goes down. Plasma norepinephrine goes down. The baroreflex-cardiovagal gain goes down tremendously. And these patients still have roughly normal fractional increases in norepinephrine during standing, and they don't have orthostatic hypotension yet, but I wouldn't be surprised if they develop it.

Well, what causes the dysautonomia in familial Parkinson's disease? Well, obviously, I don't know, but one cause certainly is synucleinopathy. And the reason I say that is we had an opportunity to study a patient who is part of this large Greek-Italian kindred, whom many of you know about, where Parkinson's disease is transmitted

as an autosomal dominant trait due to mutation of the gene

that encodes alpha-synuclein. And one of these patients was studied at the NIH.

We found that he did have orthostatic hypotension. He had never been diagnosed as having orthostatic hypotension until he came to the NIH where somebody measured his pressure lying down and standing up. The orthostatic hypotension was from sympathetic failure because of the abnormal baroreflex response. The problem with phase II, lack of overshoot in phase IV. And here you can see that although the perfusion of the heart was okay, the sympathetic innervation of the heart was essentially gone.

So at least one cause of the dysautonomia in familial Parkinson's disease is the same cause as the cause of the movement disorder which is the synucleinopathy.

So in conclusion, cardiac sympathetic denervation characterizes both familial and sporadic Parkinson's disease. The denervation appears early. It progresses over time. Neurogenic orthostatic hypotension in familial Parkinson's, as well as in sporadic Parkinson's, is associated with more generalized noradrenergic denervation, and baroreflex cardiovagal failure. And finally, alpha-synucleinopathy can cause

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both a dysautonomia and the movement disorder in familial Parkinson's disease.

And I want to thank the members of my neurocardiology team. For those of you who were up late watching the Yankees, you recognize Yankee Stadium here. Courtney Holmes is our star short stop. Sandra Brentzel is the one who refers all the patients, so she's at a pivot position. Teresa Jenkins is our patient care coordinator. She's the one who catches all the calls from the patients and their physicians. Irv Kopin, who was the scientific director of our institute for 11 years, now is in a scientist emeritus position, so he's in the coaching box. And I'm the one who gets to give the pitch.

(Laughter.)

DR. GOLDSTEIN: Thank you.

(Applause.)

DR. GOLDSTEIN: The next speaker is Sonia Peltzer. Sonia is the President and founder of FD Hope and I think will provide a very different perspective about research and the future of research into mechanisms and treatment of FD.

DR. PELTZER: Thank you, Dr. Goldstein. I'd like to thank particularly Katrina Gwinn-Hardy for all her help in making this whole conference a reality.

I'm going to first introduce myself and tell you

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a little bit about myself. I'm here for three reasons. The most important reason is I'm the mother of four children. My oldest 9, Benjamin; my second is a 7-year-old Rachel. And they asked me to say something about them, so I'd like to say that it is because of their generous sharing of their mother that I'm here today. And

I have a 4-year-old son, who will be 5 on Sunday, by the name of Samuel. Samuel has familial dysautonomia. I also have a 3-year-old daughter named Sarah who will be 4 in December, and she also has familial dysautonomia. That is why I'm here, and in some small part that's why we're all here.

I'm also here because, as Dr. Goldstein mentioned, I'm one of the founders of FD Hope, and I'm the current President. Familial Dysautonomia Hope is a nonprofit organization that was founded in the early months of 2001. Almost immediately upon founding, we initiated contacts with the NIH because one of our major goals was to get a conference like this going. Our mission is to expand research, accelerate research to find a cure, and that's what you're going to hear about from me today, encouraging more of that.

Almost 20 years ago, I was graduate student at Johns Hopkins studying these. These are cuneiform tablets. There are thousands of them. Many of them have

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yet to be translated. This particular one is about the Gilgamesh epoch for those of you who know about that. And I sat in my little cubicle looking at my Acadian language studies, and I thought to myself one day if I never translate these tablets, if no one ever translates these tablets, will it ever make a difference? And the answer was a resounding no. Within three months, I had left graduate school and had planned to go to medical school.

You can make a difference and that's why I'm sure most of you have gone into the fields that you have.

One way to make a difference, whether you're a basic scientist or a clinician, is to do translational research. The key to translation is mentoring a new generation of translational researchers not only for you to do research but for you to encourage those who follow you to do translational research.

And this is the definition of translational research that I got from the Scientist, which came out just this past week. Essentially it's research that goes from the lab to bedside and then back to the lab bench.

This morning we heard a lot about possible mechanisms behind some of the clinical symptoms of familial dysautonomia, and I'd like to talk a little bit about that.

The bottom line is patients are dying. Children

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are dying of FD. Adults are dying of FD. They're dying because of cardiovascular complications, heart attack, stroke, dysrhythmias. You expect heart attacks in your 60's. These are 20-year-olds. Renal failure, pulmonary complications, not just the aspiration but the restrictive lung disease caused by scoliosis which fortunately, because of the interventions introduced by Dr. Axelrod and Dr. Maayan, the surgeries, et cetera, we're seeing less of. Baroreceptor dysfunction. Patients are suffering.

Autonomic crisis Dr. Axelrod touched on briefly and we got a list of some symptoms, and I'll do the same

thing. I'll give you a list of symptoms. But I want to give you a true sense of what autonomic crisis is, and I'm going to describe briefly what my daughter Sarah goes through when she goes into crisis.

Sarah is, as I mentioned, 3. For a year-and-a-half, she was in crisis once or twice a month. I started her on some different products, and she's been essentially crisis-free. She's had five episodes of crisis in the succeeding year-and-a-half, but I cannot tell you enough what a horrible thing this is.

A child who is pre-crisis, which you sometimes see before they go into full-blown crisis, isn't hungry. She might go from eating six times during the day to eating once or twice and refusing all other food. And

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this is eating through a gastrostomy tube. They sweat. They drool. They're uncomfortable. Those who have geographic tongues, the geographic tongue tends to become more prominent during the pre-crisis phase.

When she goes into crisis, Sarah will start vomiting. Now, she has a fundoplication. Depending on your philosophy, you either don't expect or do expect vomiting. When she starts to vomit, she will vomit for 12 hours. I know that you've all had gastrointestinal viruses. Imagine your very worst one and stretch it out for up to a week. That's what crisis is. She will vomit. She will sweat. She's in abdominal pain. Blood pressures are sky high. Heart rate, 150s, 160s. When she was in an ICU at one point as an infant, she had an arterial line placed, and we documented blood pressures of 240 over 160 in a 3-month-old.

This is not something to be taken lightly. Children die because of crisis, and those who don't, I've heard parents say their children wish that they were dead. This is a horrible, horrible disease.

When Sarah starts to come out of crisis, she'll throw up for about 12 hours and then she'll lay there and be miserable. And part of crisis symptoms are these personality changes. They cry. They're unhappy. Nothing makes them happy. Their abdomens hurt. She will go into

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a period of half awake/half asleep, almost an encephalopathic picture, not completely out of crisis. You can't push more than 5 cc's. And for those who don't know what that means, it's a teaspoon of saline type solution through her G-tube every 5-10 minutes. And you're up for 24 hours a day, 7 days a week until she goes out of this period unless you're in the hospital to get IV fluid.

From there, she will eventually go into what's called the restorative sleep, and when she starts to sleep, we all breathe a sigh of relief. She will sleep -- and the longest we've documented in Sarah -- and other children have different amounts -- was 27 hours straight. And we were thrilled. She comes out of it, bounces back as if she had never been sick.

I'm going to show you a video that will show you

what crisis is like. It shows a child actually in the throes of crisis.

(Video shown.)

DR. PELTZER: As a physician, I'm going to go through all the list of symptoms that you see, cardiac symptoms, the high blood pressure, the tachycardia, cold extremities, mottling, purple mottling of the extremities, and blotching, GI symptoms, retching or vomiting, retching when you have really tight fundoplication, inability to

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swallow or to talk, abdominal pain, increased gastric acid, gastric secretions. You get gastritis and ulcers. Hypersalivation and drooling. We also know the bronchorrhea as well. Increased reflux and impaired esophageal motility.

The interesting thing is that when a child is in what I term, for lack of a better term, pre-crisis, a child cannot be in crisis but for up to a month be not quite right either. You will see an increase in reflux in those children. You see decreased swallowing ability and loss of some speech ability as well. They're not in crisis, but you still see these early crisis symptoms.

Constipation, gaseousness. Renal. You can see the urinary retention. Headache, emotional ability, feeling panicky.

I described my daughter's crisis. My son's crises are very, very few and far between. I can count on one hand the number of times he's been in crisis, and some of those have been only an hour at a time. But he is wild. He cannot lay still in bed. He just goes from one side to the other. A totally different picture of crisis in two siblings.

Thrashing, moaning, lethargy, agitation, being out of it. These are all words written by parents about children with FD. Fever, chills. They can have normal

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temperatures and within a space of 5 minutes have a temperature of 103 and an hour later be normal again.

Dilated pupils, obviously, and the hyperhidrosis. When I say hyperhidrosis, that sounds like a very clinical term. In reality it means, if you don't put towels down on your bed, you're washing about five sets of sheets during a crisis day. They absolutely soak through. You can literally take a shirt off and wring it and see sweat drip off.

Rubbing the body is another sign that a lot of parents say that they see their children do before they actually go into the full-blown crisis. They'll rub their nose, their extremities. One parent says that her child will rub their dog.

Self-mutilation. There have been children who have had horrible disfiguring scars on their face. Finger biting, but scratching at the face as if they're trying to get something gone. Something is bothering them.

The severity of the problem. Well, I hope I have described for you how significant this problem is.

A year-and-a-half ago, we did a survey of

families, a patient-based survey on symptoms, and we did particularly the gastrointestinal symptoms prior to going to the GI conference, the DDW conference. One of the questions we asked was about crisis. We had 54

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respondents. 13 of 16 respondents that did answer this question on impact on quality of life gave crisis a 10 out of 10 in severity. 85 percent of the 55 patients have had autonomic crisis, and our numbers, it was 45 percent of them had crisis at least monthly, and that's daily, weekly, or monthly. 17 percent of the patients who experienced crisis have it daily. Now, for those patients, it may not be quite as full-blown as I had described. They may have a crisis period in the morning. Unfortunately, the treatment for crisis is medications that are pretty sedating. So even though you may be out of crisis, you've lost your day.

Aspiration pneumonia and pulmonary damage. You see regression of developmental milestones during that period.

Another thing is impact on lifestyle. Like I said, these drugs are sedating.

And the piece that I cannot describe enough is the impact on the family. We think of diseases as affecting individual patients. This disease, as most diseases, affects families. Parents can't go away. My husband and I have been out together four times in the past five years. You cannot get out of the house unless you have someone you can trust with your children. And this disease is so unpredictable and devastating, you

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cannot trust just anyone with your children. It is the unpredictability of crisis that is just so horrible.

There is an individual with familial dysautonomia who goes into crisis when she urinates, not all the time, but sometimes. So this young woman, who's in what should be the prime of her life, rarely goes out of her house.

Things that can trigger. I'll just put these all up. These are some of the triggers of autonomic crisis again from the patient family survey, as well as adolescents.

There are things that can be done by all of you. You're here because you have a lot of experience in your respective fields and you're very talented. It is my hope that when you leave today that you're going to go back to your bench, if you do research, with a goal in mind, and that is to make a difference in patients' lives. Translational research is not just a buzzword. It's an essential form of research today.

We heard this morning from Dr. Hilz that heart rate variability decreases with Valsalva. There have been several studies looking at alpha-lipoic acid in diabetics with cardiovascular dysautonomia, and they suggest that there is improvement in heart rate variability. An alpha-lipoic acid is an antioxidant. Taking that clinical

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piece, applying it to FD, and then going back to the lab to say, if it does make a difference, do antioxidants impact on heart rate variability and why. That's the kind of research that I charge you all to do today.

The need for translational research. We need both clinical as well as genetic research, and clinically significant research that's going to address the areas with the greatest impact are things that will impact on blood pressure control, hypertension, as well as orthostatic, renal protecting, cardiac dysrhythmias, autonomic crisis, preventing crisis, what are some common mechanisms behind those triggers, as well as new treatments.

Genetic research that will have impact. We've heard a lot about these, obviously. I'm not a geneticist. I'm not going to go over this in detail.

Even the smallest person can change the course of the future. You all are not small people. You're big people. Please change the course of our children's future.

I'm going to end with some photos. This is Michele. These are all children with FD. Dovi, Evan, Michele, David. David Rancer is no longer with us. He died a year ago of malignant hypertension we believe. Rebecca, Zack, and this is my own, Sammy and Sarah.

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Our future is in your hands. I implore you not to be lured by the fascination of science for science's sake. If you apply your talents towards translational research and encourage those who come under you for training, you can save a life. You can save lots of lives.

So because FD is a Jewish disease, I'm going to end with a quote from the Talmud. If you save a life, you save the world.

Thank you.

(Applause.)

DR. GOLDSTEIN: I told you it would be different, and it will continue to be.

The next speaker is Dr. Ando who's an associate professor and Vice Director of Laboratory Medicine at Kumamoto University School of Medicine in Japan. Dr. Ando is an expert on a different type of inherited dysautonomia, familial amyloidotic polyneuropathy. I'm very interested in hearing what he has to say about this other condition, also rare, but I think you'll see some rather striking similarities in the issues and the strategies scientists have tried to employ to understand and treat this condition.

DR. ANDO: Thank you very much, Dr. Goldstein.

First of all, I would like to express my

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gratitude to the organizing committee of this workshop for giving me a chance to talk about our original research on FAP and autonomic dysfunction.

FAP is amyloidosis and amyloidosis is a disorder of protein metabolism in which soluble proteins are

deposited in the tissues as abnormal insoluble fibrils. Amyloid fibrils are like nylon fibrils. In other words, amyloidosis is similar to nylon storage disease.

Familial amyloidotic polyneuropathy, or FAP, is an incurable hereditary amyloidosis and transmitted via autosomal dominant mode of inheritance.

The precursor protein of amyloid deposition in FAP is a mutated transthyretin which is predominantly produced by the liver and binds to T4 and retinol-binding protein in plasma.

About 100 point-mutations or deletions have been reported, and most of them lead to FAP. Of those, 20 show mainly autonomic dysfunction.

This slide shows amyloid deposition in the nervous system in FAP patients. Amyloid deposits are commonly found in sympathetic and dorsal ganglia and in peripheral nerves in addition to systemic organs.

Of the various types of FAP, the FAP TTR methionine 30 type is the most common. The clinical manifestations are classified into three major groups.

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Among them, those of the autonomic dysfunction group have the greatest effect on the patients' lives because they restrict their daily life.

The duration of FAP, after the onset of the disease, is about 10 years. In addition to various autonomic dysfunction, severe systemic organ dysfunction is commonly observed.

Until 10 years ago, the foci of FAP Met 30 were restricted to several endemic geographic areas. However, owing to the progress of molecular genetics and protein chemistry, now this disease is considered to be a worldwide occurrence. This is a map of Japan. In addition to two major foci of Met 30, various other types of FAP have been discovered in many districts of Japan.

To evaluate autonomic dysfunctions found in FAP patients, we employed those, some of which we originally developed.

In terms of autonomic dysfunction, orthostatic hypotension causes FAP patients to have terrible problems. The trauma to this patient's left eye was noted when he tumbled down after he stood up. We recently reported the interaction between cerebral blood flow and autonomic dysfunction. As you can see in this slide, the reverse flows in common carotid artery and vertebral arteries are always noted when the patient stood up and experienced

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clinical symptoms such as syncope and a feeling of faintness. No reverse flow could be detected when the clinical manifestations were absent.

As shown in this slide, the clinical manifestations correlated with the presence of reverse flow. However, no correlations between the degree of orthostatic hypotension and the reverse flow, the presence of reverse flow, were observed.

I would like to talk about the disturbance of peripheral circulation in FAP. Various clinical symptoms

reflecting autonomic dysfunction are commonly found in FAP patients as initial symptoms. However, it was not yet known when autonomic dysfunction starts in FAP patients. So to elucidate the question, we applied laser Doppler flowmetry to measure the change in the blood flow in the phenotype. As shown in this slide, in control subjects a significant decrease in the blood flow was detected after deep inspiration. In contrast, in the FAP patients in the advanced stage, no such responses were sighted at all. Interestingly, some asymptomatic carrier of the Met 30 gene showed poor responses for the stimulations. These results suggest that peripheral or sympathetic C fiber may be first impaired prior to the sensory and the motor nerves.

We could detect abnormal vessels microscopically

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in the conjunctival vessels in FAP patients interestingly, as shown in the slide. _____ of the vessels and micro_____-like changes, these, could be found in most of FAP patients. Moreover, these changes could be detected in patients with pan-dysautonomia and Shy-Drager syndrome. These results suggest that these abnormal vessels can be formed via autonomic dysfunction.

We could detect abnormal venules in the finger of FAP patients, as shown in this slide, by near-infrared spectroscopy system. These abnormal vessels could be detected in most of FAP patients.

As you know very well, cardiac autonomic dysfunction can be evaluated by two methods as shown in the right-hand side of this slide. In the advanced stage of FAP patients, neither low nor high frequency component could be detected at all.

MIBG accumulates in the peripheral sympathetic nerves of myocardium. However, in the advanced stage of FAP patients, no such accumulation was observed.

Next I would like to talk about disturbed glandular secretion in FAP patients. This slide shows 75 gram OGTT pattern in FAP patients, as shown in this slide. Significant hypoglycemia followed after hyper-insulinemia. Interestingly, some FAP patients showed hypoglycemia.

We also examined the secretory function of

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salivary glands and lacrimal glands. This slide shows amount of salivary secretion in different FAP patients. Right is FAP patients and left is healthy volunteers. Although the total amount of salivary secretion was very low in the static state, after they chewed the gum, the _____ hypersecretion of saliva was detected in several FAP patients. As shown in these slides, the same phenomenon could be detected in tears secretion after topical installation of pilocarpine. These results suggest that denervation super-sensitivity occurs not only in the exocrine glands, but also in the endocrine glands. It is very important to note the dynamic aspect of secretory function in these glands.

This slide shows increased postprandial hypotension and hypoglycemia after taking a meal. 60

minutes after taking a meal, increased postprandial hypotension and hypoglycemia were observed in this patient.

To elucidate the disturbed glucose metabolism in FAP patients, we performed a histochemical analysis for autopsy of FAP patient samples. As shown in this slide, although the Langerhans islet itself remained intact, significant amyloid deposition was observed in the pancreatic stroma, enteropancreatic nerves, and around the vessels. These amyloid depositions can be the results of

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denervation hypersensitivity of glucose metabolism.

Next, I would like to talk about the gastrointestinal motility in FAP. In most FAP patients, drugs given by oral administration sometimes cannot express their maximum effect. Therefore, we synthesized a nasal application of L-threoDOPS, the drug for orthostatic hypotension, with a _____ mixture. With this application, the plasma norepinephrine levels, the converted form of L-threoDOPS, was significantly elevated 2 hours after application. However, no such increase was observed with oral or vehicle alone application.

To evaluate the gastrointestinal motility in FAP patients, we applied electrogastrography. In healthy volunteers, the motility of 3 _____ per minute were usually observed. And after taking a meal, the increase gastrointestinal motility could be detected for more than 30 minutes. In contrast, in FAP patients with severe diarrhea, after taking a meal, the increased gastric motility continued for more than several hours. In patients with constipation, no such increase was observed.

By the way, anemia is one of the very common clinical manifestations in FAP. Recently we have reported the correlation between anemia and autonomic dysfunction in several neurological disorders. In FAP patients, significant correlation between two factors were observed.

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In contrast, in ALS which have no autonomic dysfunction showed no anemia.

This slide shows 6-hydroxydopamine-induced anemia in rats. 6-hydroxydopamine damages the peripheral sympathetic nerve terminal, resulting in neurogenic anemia. That was reversibly treated by simultaneous administration of desipramine, a competitive inhibitor of 6-hydroxydopamine.

To treat the anemia found in FAP patients, we administered human recombinant erythropoietin because the anemia commonly found in FAP patients is usually a normochromic, normocytic pattern, as shown in this slide. Plasma hemoglobin levels were significantly increased, and the frequency of orthostatic hypotension was reduced.

In the remainder of my talk, I would like to discuss liver transplantation on autonomic dysfunction in FAP.

As I said before, the precursor protein of amyloid deposition in FAP is a mutated transthyretin. Transthyretin is predominantly produced by the liver, so

liver transplantation has been studied for the treatment of FAP. After liver transplantation, as shown in this slide, the variant TTR levels in serum were dramatically decreased day by day. Liver transplantation improved partially autonomic dysfunction. This is typical data of

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an FAP patient 8 months after liver transplantation. And several autonomic function improvements, especially urinary function, bladder function improved greatly.

This slide shows MIBG scintigraphy before and after liver transplantation. As shown in this slide, 8 months after liver transplantation, significant accumulation of MIBG was observed in the myocardium. This suggests recovery of sympathetic nerve _____.

Erectile dysfunction is one of the most serious quality of life problems for FAP patients, so we administered Viagra to FAP patients. This slide shows I evaluated by thermography. One-and-a-half hours after administration, erectile dysfunction was treated, although no ejaculation was observed during sexual intercourse.

Because of severe shortage of donor livers, domino liver transplantation using the _____ FAP patients' liver has been performed recently. In the second liver recipient, the variant transthyretin in the serum suddenly started to produce from the liver. However, we do not know what types of autonomic dysfunction can be observed in these patients. We must wait a time.

Liver transplantation has several problems, so we must have more noninvasive therapy for FAP patients. So we moved on to the trial of genomic TTR therapy in FAP.

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We constructed three different types of single-stranded oligonucleotide having the normal TTR gene. With this SSO, we intrahepatically injected the normal gene SSO using the transgenic mice having no Met 30 gene. This is the result. Significant gene conversion from the variant TTR gene to normal gene was observed. Although this is preliminary data, we do hope the day will come when we can analyze the autonomic dysfunction in FAP patients who undergo liver transplantation.

Thank you very much for your attention.

(Applause.)

DR. GOLDSTEIN: Now Dr. Maureen Hahn will talk about another inherited dysautonomia that you'll be hearing about in other talks, but this is the first mention of it in this meeting, and that is dysautonomia from deficiency of function of the cell membrane norepinephrine transporter. Thank you.

DR. HAHN: I'd like to thank the organizers for allowing me the opportunity to speak today, and I'm going to tell you a little bit about some of the work I've been doing in the laboratory of Randy Blakely at Vanderbilt University.

In a little bit of a switch from some of the other talks we've heard, we're actually not a dysautonomia lab, but we're a laboratory that studies monoamine

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transporters, one of those being the norepinephrine transporter. The norepinephrine transporter is one of a family of proteins that are localized presynaptically to plasma membranes of sympathetic neurons for one and all noradrenergic neurons, so those in the brain as well.

The function of the transporter is to take norepinephrine back up from the extracellular space after it has been released by exocytosis by a neuron. This is a sodium-dependent process. And once the norepinephrine is taken back up into the cell, it can be repackaged into vesicles for further release, or it can also be degraded by monoamine oxidases to the metabolite DHPG, which I'll mention a little bit later.

The transporter was cloned in 1991 by Randy Blakely, the cDNA for it, and the cDNA codes for a 617 amino acid protein which has 12 putative transmembrane domain spanning regions, as well as intracellularly localized amino and carboxy termini. There are potential sites for phosphorylation, and in the second extracellular loop, there are sites for glycosylation, which is something that I'll touch upon again later in the talk.

This slide is just meant to illustrate to you what I've said, which is that the norepinephrine transporter, or NET, is localized to synapses of the sympathetic nervous system.

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This slide shows a culture of superior cervical ganglion, and on the left, you're seeing staining for FM1-43, which is a dye that is taken up after exocytotic release of neurotransmitter, and so it labels for you sites of neurotransmitter release. On the right we can see the same section co-stained with an antibody to the norepinephrine transporter, showing you that the transporter is localized at sites of release. So this emphasizes the importance of this protein in regulating reuptake of norepinephrine and thereby contributing to norepinephrine homeostasis in the body.

I just want to also mention that transporters, including the norepinephrine transporter, are targets for multiple psychoactive drugs, including some that have been mentioned already and some that have not. The norepinephrine transporter is a site for binding for cocaine, amphetamine, MDMA, or ecstasy, antidepressant compounds, or methylphenidate, also known as Ritalin, the drug used for attention deficit disorder. This also emphasizes the importance of this protein and that genetic variation in this gene, which is something that we're interested in, could not only influence norepinephrine homeostasis but the response to various drugs.

So we have been in our laboratory looking for genetic variation in the norepinephrine transporter and

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its possible role in the contribution to disease. This slide just shows you that the gene has been cloned and localized to chromosome 16. And below that you see the organization of the gene which has 16 identified exons

which, when transcribed and translated into the protein, give you that protein with 12 transmembrane domain structure that I showed you. In addition, two splice variants have been identified, which I won't talk about today, giving further opportunity for genetic variation to influence expression of the protein.

One thing I want to emphasize is that NET is a single-copy gene which would indicate that if there is genetic variation or if the gene were deleted, that there would not be a lot of opportunity for compensation. This is the only protein that is responsible for the high affinity uptake of norepinephrine back into the sympathetic neurons.

This slide is a little bit more to remind me than to remind you because we have a lot of dysautonomia experts in the room, but the disease that I'll be focusing on today is classified as a dysautonomia, and I'll be talking about orthostatic intolerance which is also gone by many names, orthostatic tachycardia or postural tachycardial syndrome, or POTS. And it was probably first identified and called Soldier's heart over 100 years ago.

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Some of the symptoms are listed here. So these are all symptoms that, as the name implies, arise when assuming an upright position for the most part. These include lightheadedness, palpitations, pre-syncope, and dizziness. Syncope can occur. Many of the patients experience fatigue, and there's an orthostatic tachycardia of greater than 30 beats per minute. So the heart rate increases and stays elevated when the upright position is assumed. For orthostatic tachycardia, this is not accompanied by an orthostatic hypotension. And plasma norepinephrine levels exceed 600 picograms per ml in patients who were diagnosed with orthostatic tachycardia. And many of the symptoms are consistent with decreased cerebral blood perfusion.

Now, orthostatic intolerance, or orthostatic tachycardia, is a syndrome that can accompany various diseases, and as such, you can imagine that there are many ways to get orthostatic tachycardia. There are many underlying pathophysiologies. As an example, an autoimmune disorder in which the body makes antibodies to a subunit of the nicotinic receptor can result in symptoms of orthostatic tachycardia as well as hypovolemia that can be caused by some chloride channelopathies that are known to exist can result in an orthostatic tachycardia.

But what we focused on in our lab and what was

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of interest to us was that a lot of orthostatic intolerance patients seem to have a very hyperadrenergic state that is primarily responsible for the orthostatic tachycardia. There are several ways to get to this endpoint. You can have increased release or increased activation of the sympathetic nervous system that's in overdrive and is responsible for the increased norepinephrine. And from what I've told you about the norepinephrine transporter now, you can also imagine that

a way in which you could get to this endpoint would be to have decreased clearance of norepinephrine following release or NET deficiency.

In collaboration with David Robertson's group at the Autonomic Dysfunction Center at Vanderbilt, one such patient was identified who had orthostatic intolerance and also seemed to manifest these symptoms that were consistent with not just hyperadrenergia but specifically a NET deficiency. One of the symptoms that was observed was that when going from a supine to an upright position, the norepinephrine levels would increase greatly, but the levels of the metabolite DHPG did not change much between supine and upright. And as I alluded to earlier, DHPG is the metabolite that is formed from the norepinephrine that is taken back up via the transporter. So if norepinephrine is not taken up via the transporter, then

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DHPG levels will not rise following release from the sympathetic nerves. So this suggested a NET deficiency.

Further evidence of this was that the compound tyramine did not seem to have much of an effect in this proband with OI. And tyramine is a compound that also acts at the transporter. I didn't mention it earlier when I talked about drugs that interact with the transporter. Tyramine is actually a substrate. It's taken up by the transporter and causes norepinephrine to actually come out of the vesicles where it's being stored for release. Once the norepinephrine is in the cytoplasm, the transporter can actually work in reverse, and you can get norepinephrine efflux from the cell. So in a normal individual, tyramine actually causes a rise in plasma norepinephrine, and in the proband in this study, plasma norepinephrine did not rise very much in response to a tyramine infusion. So this was also suggestive of NET deficiency.

In fact, when the DNA from this patient was directly sequenced, there was in fact found to be a mutation in the transporter, and it was a single-base substitution that changed the three nucleotide code that codes for the amino acid alanine and converted this to a proline. You can see from the sequencing chromatogram that there are two peaks here where there should actually

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be one, as you see in the surrounding peaks, and that indicates a change in the nucleotide. And because there are two peaks there, that's also indicating that this is a heterozygous mutation. There actually is one normal sequence at that site and one allele with the mutation.

Again, showing you the schematic of what the transporter topology looks like, this mutation, for the alanine to proline switch, is located at amino acid 457 which would put that in one of the transmembrane domains of the transporter, or TMD 9. Introduction of a proline is known to cause bends in the helical structure of proteins, and so you can imagine that this would have the potential to influence the protein function.

When a pedigree was generated for the patient's

family, you can see that we were able to generate a pedigree showing, within three generations, the heterozygous presence of this mutation in several family members. In fact, when the entire family was looked at for phenotype related to this, it would seem that if we divide the family members into those that have alanine and alanine on both chromosomes, so no mutations, and compare them to the alanine and proline, if we look at the mean heart rate, we can see that supine, there's a trend for an increase in the heart rate in those family members with the proline mutation and that upon standing, there was a

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significant increase in the heart rate in the family members with the proline mutation. So it did seem to track with the phenotype in the family.

So what we then did was to create the mutation in the lab and, using an expression vector, expressed the mutation in cell lines in the lab and looked to see what the function of the mutant to norepinephrine transporter was.

If you just look at A, what I'm showing here was the ability of the transporter to take up the substrate norepinephrine. So we see on the y axis is a tritiated-labeled norepinephrine that is taken up over concentrations of norepinephrine shown on the x axis. You can see here this top curve represents the normal or the wild type transporter and how you see saturable kinetics of uptake of norepinephrine overall concentrations. If we compare that to the A457P mutation, we see that there is very little, if no uptake at these concentrations of norepinephrine.

We also then looked with a radioligand binding, RTI55 is a compound that's similar to cocaine and binds to the transporter. We used this to get an idea of protein binding levels in the cell. So in membrane preparations from the cells that are expressed in the transporter, we'd look and see that over increasing concentrations of

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RTI-55, we get again saturable kinetics of binding for the wild type transporter and we get binding to A457P, but it appears to be much lower compared to the wild type.

We also looked, as you'll see in C, at the ability of A457P to bind the substrate norepinephrine. We knew it wasn't transporting from the experiments we did here, and we were curious to know if the binding site had changed. In this experiment, we're competing the binding of the radiolabeled ligand with increasing concentrations of norepinephrine, shown on the x axis. We can see that the wild type is the curve to the left, and we can see that norepinephrine is able to compete off the RTI-55 and that the A457P curve, which is shown to the right, has shifted to the right, indicating a decrease in the ability of norepinephrine to compete off the radioligand, suggesting that it had lost some affinity, although it does seem to bind to the transporter.

So we knew that we had, therefore, a transporter that had some -- we knew there was some protein expression

from the membrane binding and that it had some ability to bind norepinephrine, although there was really a complete lack of transport. So we decided to use Western analysis with specific antibodies to the transporter to look at the actual protein expression in these cells.

A shows you a typical Western blot analysis.

What you see on the left is extracts from cells that are run on a gel and then blotted with an antibody that binds to the transporter. And in untransfected cells, you see no signal. And if we look at the hNET wild type, you see several dark bands on this gel. These are all norepinephrine transporter, and there are several different sizes because, as I mentioned earlier, the protein has the ability to be glycosylated. What we've shown in our lab previously is that these lower bands around 56 kilodalton are a glycosylated form that happens initially, probably in the ER, and then this higher molecular weight form, between 80 and 100 kilodaltons, is a further processed glycosylated form.

If we look at the hNET A457P, we see that there's a lot of this lower form, but there really is a great decrease in this higher molecular weight form. So although the A457P is making protein, it seems to not be making this higher glycosylated form as much as the wild type.

On the right, where I have surface expression, we've used cell impermeant biotinylating reagents which can label the surface proteins of a cell, and then we collect those on streptavidin beads, and we can look and see how much of that transporter that was made is actually on the surface of the cell because we know we have a lack of transport and we're trying to understand the reason why.

You can see in the wild type NET that you get a large amount of the 80 to 100 kilodalton form on the cell surface. And we've shown in our lab previously that this is the case, that this larger form, or the fully glycosylated form, is the form that's expressed on the surface and is, therefore, responsible for the transport of norepinephrine.

And the A457P again is diminished in this 80 kilodalton form, as it was in the totals, but this just emphasizes that that form is, in fact, diminished on the cell surface. And that's just graphically represented in B that there's a decrease in the mature form of the protein, both in the totals and on the cell surface.

So what I've told you thus far is that if we compare in C, if this is just wild type activity in the blue bar, and we compare various features of A457P to that, you can see that we have a decrease in the amount of protein present, as indicated by binding, Western analysis, and wholesale binding, which I didn't show today, and that even though there is some protein that is being expressed on the surface of the cells we've measured by these biotinylation studies, even that protein is

inactive because the uptake is much less, less than 1 percent, virtually not there.

So being we had a protein that was -- some of the protein was present, although it was a nonfunctional transporter, we became very interested in whether or not we would see a dominant negative interaction of the mutant on the wild type protein. Just by way of review, a dominant negative interaction would mean that the mutant not only loses its function but interferes with the function of the normal allele that is present. So this would be very important in a disease with features of heterogeneity.

Now, a dominant negative should, therefore, cause a more severe phenotype than if the gene were completely deleted or the protein not expressed at all. So if it wasn't there, it wouldn't be able to interfere with the normally functioning allele.

Finally, a physical association of the normal and the mutant protein forms is a potential mechanism for this to occur. So the way in which a mutant protein could influence the wild type function would be if they existed in some kind of complex together and the mutant couldn't interfere with the wild type.

And in order to examine this, what we did was we put tags in the amino terminus of the protein, tags that could be identified with unique antibodies because, remember, the mutation that we have here is only different from wild type NET by one amino acid. So in order to look at the wild type and the mutant together in the same cells to look for dominant negative interactions, we have tags in the amino terminus, either a His tag or an HA tag that can use different antibodies.

What we found when we did this was that if we co-expressed the mutant and the wild type together, which is shown here at the bottom of the graph -- that's just the amount of DNA that is transfected into the cells, and it's either wild type or A457P. And the y axis is showing you the tritiated norepinephrine uptake. You can see that we have a certain amount of uptake for the wild type transfected alone, and when we transfect that with the mutant, there is actually a decrease in the uptake compared to that same amount of wild type transfected alone. And the only difference now is the presence of the mutant.

And in part B, we see another Western blot analysis that mirrors the experiment in A. The wild type here is labeled with that HA tag, and now we use an antibody that only recognizes that tag on the Western. And so what we're looking at here is the wild type expression and not the mutant, wild type alone. And we're looking at it in a biotinylation experiment. So we're looking at what's on the surface of the cell.

What we can see here is that under the same conditions, wild type expressed alone gives you this

amount of protein on the surface, and when it was co-expressed with the mutant, in fact, the wild type expressed on the surface actually decreased.

And this next lane on the gel shows you that when we transfect twice the amount of wild type, that we actually do get an increase in wild type, when we double it, compared to this first lane. And that's graphically represented here, showing you that again the mutant can decrease the wild type surface expression and that transfecting even more wild type in does increase the surface expression. And that indicates to us that we're not just saturating either some machinery in the cell for making the protein or trafficking it because we can get more expression when we double the amount of DNA. So this said to us that there was, in fact, a dominant negative interaction.

Next we wanted to look at whether or not there was a physical association between the proteins. And it's been shown for many G-protein coupled receptors and also recently it has been shown for transporters in this gene family that the proteins do exist in oligomeric complexes. So our next experiment was to look to see if NET existed

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in a complex, and one way to do that is to try to co-immunoprecipitate the two proteins. So, again, you're using differentially tagged NETs, and if one tag precipitates the other, then the proteins are together in a complex.

So what we did here was to, again, transfect cells with the DNA shown on the bottom of the graph. The first two lanes just show you controls, showing that either when the wild type or the A457P was transfected alone separately and subjected to immunoprecipitation, there was no signal on the blot which was to be expected.

When the two differentially tagged His hNET or HA hNET were transfected together -- and I'm just showing you wild type in this gel. I'm sorry. I forgot to indicate that. The evidence of NET in a complex hadn't been shown before. So prior to looking at the mutation, we needed to look at the wild type. What in fact we saw was that we could co-immunoprecipitate the protein.

And I'll just quickly show you that in part B. When we repeated the experiment, if we look at the immunoprecipitated lanes, the first lane showing wild type/wild type and the second lane now showing mutant and wild type interaction, in fact the A457P can immunoprecipitate the hNET wild type, showing that these could exist together in a complex and be responsible for

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the dominant negative effects that we saw.

And that's just all summarized here. The important take-home message from this really is that individuals that have a heterozygous mutation for hNET, such as the OI family, might actually have less than 50 percent of the normal NET capacity if dominant negative mutations are involved.

And I'll just end there and I won't have time to

read the people, but to acknowledge the people who have contributed either in our lab or in David Robertson's group to this research. Thank you.

(Applause.)

DR. GOLDSTEIN: Of course, in between alleles and genetic mutations are polymorphisms, and I think there's been an explosion of information about genetic polymorphisms. This certainly applies to autonomic regulation, and Steve Liggett will give us an update about that.

DR. LIGGETT: Thank you. I'm going to go ahead and get started while things warm up here.

I want to make sure we're all on the same page here in terms of definitions. A polymorphism I'm going to define here as genetic variant which occurs at a frequency of 1 percent or greater in the population. This really differs from what we've been talking about over the last

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few hours which are mutations which are very uncommon and typically are sufficient to cause the disease. In contrast, polymorphisms are occurring throughout the population of apparently normal individuals. They're present in this room amongst us, and the intriguing concept is that they may modify diseases or perhaps in concert several of them that have significant impact on a protein function together might lead to a substantial risk of a disease. But individually they may not be such powerful predictors.

So today I want to go over with you the work that we have done on adrenergic receptor polymorphisms. Now, why have I picked that? Well, I think that's pretty obvious that the adrenergic receptors, both the alpha 1 class, the alpha 2 class, and the beta adrenergic receptors, are all critical for homeostasis and are a major limb in the sympathetic part of the autonomic nervous system which has already been described.

So in doing these types of studies, I want to emphasize that we have been very careful, as shown by the previous speaker, not to simply find a single nucleotide polymorphism, sometimes called SNP, and then go do an association study and then be happy if we see some sort of weak association. We think that's bad science. Our approach has been rather, if we find a polymorphism, to

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build it, put it into cells, study its pharmacology and its biology, and if in fact there is a difference in the pharmacology, then we'll pursue it in an appropriately hypothesis-based clinical study.

So these are the receptor subtypes that I'm going to talk about, and I want to just quickly remind everyone of the coupling pathways. The alpha 2 receptor is coupled to GI. They inhibit adenylyl cyclase. The beta receptor is coupled to GS. They stimulate adenylyl cyclase. There are three members of the beta receptor family. There are three members of the alpha 2 receptor family. There are three members of the alpha 1 family which couple to GQ and couple to phospholipase C.

Interestingly enough, there is one polymorphism in the alpha 1B receptor. We don't know about these two, and this doesn't appear to do anything. So I'm going to concentrate on these two families in particular. I thought the best way to approach this was to simply march through the receptors and show you what their phenotypes are, not spend a lot of time on why the particular polymorphism does what it does -- those are in the papers -- and then show you a straightforward hypothesis-based study which will show you the power of polymorphisms, particularly when they are in combination.

So the alpha 2A receptor is a centrally

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localized receptor also found in the peripheral sympathetic nervous system and there is one polymorphism that we have found that is very rare. It occurs in .4 percent of whites and 4 percent of African Americans. And it is a substitution of lysine for a spare gene at this position in the third intracellular loop.

The phenotype of this receptor, when put into Chinese hamster ovary cells, is interesting in that it's a gain of function. So individuals who have this receptor would have an increased function of the alpha 2A receptor. That's shown here as an increase in the ability to inhibit adenylyl cyclase. And this is both with the endogenous catecholamine as well as a synthetic drug.

Now, interestingly, the signals sometimes are much greater -- the phenotype is much greater depending on which signal you look at. So the alpha 2 is also coupled to MAP kinase, and you can see the gain of function with this lysine 251 polymorphism is actually quite striking when you look at this signal as compared to inhibition of adenylyl cyclase.

Nevertheless, this is relatively rare, and in the work that I work with hypertension, heart failure, and asthma, it probably wasn't going to be a player.

Now, to the alpha 2B's. These receptors are localized both centrally and peripherally as well. Here

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the allele frequency is 31 percent in caucasians and 12 percent in African Americans, and it consists of a deletion of three glutamic acid residues right in the middle of a known region of phosphorylation by G-protein coupled receptor kinases which are important for desensitization of the receptor.

I will tell you this is actually a 7 transmembrane receptor, but for clarity I'm just showing the areas where the polymorphisms occur.

So for this polymorphism, when these were expressed, these receptors were expressed in CHO cells. You can see that in fact we have a minor decrease in the ability to inhibit adenylyl cyclase with the polymorphic receptor, fairly small, a little rightward shift in the dose-response curve, but not very impressive.

But as I said to you before, this area is very important in the receptor for phosphorylation by G-protein coupled receptor kinases which is involved in agonist-

promoted desensitization. So we looked at this particular property and found that wild type alpha 2B receptors undergo about a 50 percent desensitization after 15 minutes of exposure to norepinephrine in the culture dish, whereas the deletion polymorphism receptors do not at all. In fact, the phosphorylation of the receptor when purified using epitope-tagged receptors is substantially less in

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the deletion 301 through 303 polymorphic receptor.

So the phenotype of this receptor is one of some uncoupling, but particularly a lack of desensitization. And desensitization is a critical mechanism for homeostasis in most G-protein coupled receptor systems and is important for sort of integrating all these signals that are coming into the cell.

Now, the alpha 2C receptor. I will show you a lot about where this is and what it does. It's localized both centrally and in the peripheral presynaptic sympathetic nerves. There is a single polymorphism that we have found which consists of a deletion of a GAGP again in the third intracellular loop. It's sort of interesting that all known polymorphisms of the alpha 2 receptors are in the third loop of the three subtypes.

This occurs with a frequency of only 4 percent in caucasians but 40 percent in African Americans, and this is one of the biggest differences that we've seen in coding block polymorphisms of any of the G-protein coupled receptors. This receptor is markedly uncoupled.

So this is again a read-out of inhibition of forskolin-stimulated adenylyl cyclase. These are transfected equal levels in CHO cells, and you can see that there's this marked inability to inhibit. It's almost dead really. The lower we go in expression towards

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a more physiologic level, we essentially see no response at all.

Now to the betas. I know this is fast, but there's a story in the end, and one of the important things that I think we need to realize, as disease modifiers this group ought to be looking at these receptors and these polymorphisms because they have a marked physiologic effect, which I'll show you in a moment.

So the beta 1 receptor, as you know, is localized primarily on the heart, on the myocyte, responsible for increased contraction as well as rate. There are two polymorphisms. The one I want to concentrate on is the substitution of arginine for glycine at position 389. The allele frequencies are fairly common, as high as 42 percent in African Americans. And this polymorphism results in a gain of function. So the y axis here is adenylyl cyclase either presented as absolute levels, or as a percent forskolin, you can see that the Arg389 receptors are clearly sort of a hyperfunctional receptor. They stimulate to a much greater degree, either absolute levels or fold stimulation over basal. So you're beginning to see that these polymorphisms have a lot of

different phenotypes.
The beta 2 receptors I won't go into in detail.

We've published a fair amount in Asthma on these. It's interesting to note that there are four polymorphic sites in the beta 2 receptor, really only three of which occur with any frequency you can find. And most interesting I think is this Ile164 polymorphism which I have down here, having an allele frequency of 5 percent, but that's actually the allele frequency of heterozygotes. We've never found a homozygous individual -- and we've looked at over 10,000 now -- who is homozygous for Ile164.
And this receptor is, in fact, pretty dysfunctional as well. Lower basal activity of adenylyl cyclase and lower maximal stimulation. Probably since it's expressed in lungs and it's required for removal of lung water at the time of birth, the homozygous individuals do not come to term or die shortly thereafter.
From a resolution standpoint, this is pretty hard to read and it's not important, so I'm going to let it go, but just to tell you that the position 16 and 27 polymorphisms of the beta 2 receptor actually affect more the loss of receptor number after prolonged agonist exposure as compared to these short-term events that I've showed you just recently.
So I put this slide up here to now give you an idea of where I think the usefulness of these polymorphisms can be. I've mentioned before that they

might be risk factors. I've also said that they might have something to do with response to therapy because we do use a lot of adrenergic receptor agonists and antagonists for treatment.
But because of their relatively high frequency, one of the concerns has been -- and I actually got this from a fair number of classic geneticists -- this notion that, well, since they're common, they probably don't do anything, which was a little discouraging back in the early days.
I would modify that in thinking another way, and perhaps this is a little more true. If they're common, they probably have a small effect, and if they're rare, they might have a big effect. So common polymorphisms which have been allowed to exist through evolutionary time, if they have a big effect, it was for a specific survival reason. If they're sort of a negative effect overall, they probably shouldn't be common anymore.
So I'm going to give you an example, though, of where you have two fairly common polymorphisms. Both have effects in cells but, when together, can give you a major physiologic effect.
So I want to talk about heart failure, and there's a hypothesis that's generated over a number of years, both in animal and human studies, that prolonged,

persistent activation of cardiac beta receptors results in hypertrophy or heart failure regardless of what the

initial insult is. So it could be some ischemia, a little myocarditis, or hypertension, or the so-called idiopathic forms. But nevertheless, persistent activation is bad. And that's why beta blockers actually paradoxically work in congestive heart failure where you would think they might not. They tend to relieve the heart of this persistent norepinephrine that is present because the cardiac output is low and the body is trying to increase its cardiac output.

Well, it turns out that the alpha 2C receptors inhibit norepinephrine release at low stimulation frequencies in the cardiac presynaptic nerve. This is what we believe to set sort of the basal or all-day-long norepinephrine secretion. The alpha 2A's inhibit norepinephrine at high stimulation frequencies, so this would be an acute response.

Now, we also know that the beta 1 receptor is the primary cardiomyocyte beta receptor, and if you begin to think about this, we have the makings of an issue here. In mice I can tell you that knocking these two receptors out results in uncontrolled norepinephrine release and heart failure.

And I'm going to show you the mouse data now

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with the two beta 1 polymorphisms very briefly. When we built these mice, we studied them in vivo, and you can see that at 3 months, the Arg389 beta 1 polymorphism mice, which is the hyperfunctional mice, have this enhanced basal and dobutamine-stimulated contractility. The Gly mice are somewhere in between, and these are non-transgenic. So these are equally expressed.

However, by 6 months, these Arg389 mice have no response to infused dobutamine whatsoever, and this is sort of a hallmark in human heart failure, the lack of beta receptor responsiveness. Indeed, when we looked by echocardiography by 9 months of age, the Arg389 mice have a significantly decreased fractional shortening as compared to the Gly389 mice. And on microscopy, they show extensive fibrosis, myocyte dropout, and hypertrophy, no apoptosis, interestingly enough, while the Gly389 mice are indistinguishable, so non-transgenic. So we considered that Arg389 might be a predisposing polymorphism for heart failure. But, of course, that's not really the whole picture.

This is a presynaptic nerve here, and the overall hypothesis would really be that you need both this alpha 2C polymorphism and the beta 1 in order to have a substantial risk for heart failure. And the reasoning, once again, would be as follows.

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If you have the alpha 2C Del322 through 325, you have decreased function, therefore increased norepinephrine release at the synapse. At the same time, if you have the beta 1 Arg389, which by itself has increased function and would have increased response at the cardiomyocyte, but is then exposed to the increased norepinephrine as well, it would have this synergistic

interaction and potential heart failure.

So we did look at a study which is going to come out next week which looked at controls in heart failure patients. We genotyped at these two loci. We did adjust for age, sex, hypertension, and diabetes, which was really not necessary. It was very clear. And because the allele frequencies are quite different between African Americans and caucasians and we did not want to deal with population stratification per se, we carried out the analysis separately for each ethnic group. And I'm just going to show you African Americans today because that's when the allele frequencies are high enough that we had enough power to show something.

So now, our normals are squeaky clean normal, and in the heart failure patients, just for those who are interested in phenotypes, there's no doubt they have heart failure. We're not talking about partial phenotypes or sort of questionable diagnosis here. Heart failure is one

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of those things, when you see it, you know it. And their ejection fractions were around 25 percent which is quite low, and most of them had class III/IV symptoms.

So you can see there's a substantial over-representation of the homozygous deletion alpha 2C polymorphism in heart failure. Indeed, 53 percent of heart failure patients were homozygous for this polymorphism compared to only 16 percent of normals. So in fact, the odds ratio is 5.65 with this really nice p value of 0.0001. So in and of itself, the alpha 2C Del322 through 325 is a risk factor for heart failure.

What about the beta 1? Well, here we don't really see much difference in the allele frequencies, either homozygous Gly, homozygous Arg, or the heterozygous individuals, between normal and heart failure subjects. So there's no risk associated with having heart failure if you have the beta 1 389 polymorphism.

But what if you have both? So, as I've shown before, the deletion has an odds ratio of 5.65. The beta 1, there's no risk. If you have both, the risk is 10.1 with a p value of 0.004. This is, to my knowledge, the highest known genetic risk factor for what we have previously called garden variety or idiopathic dilated cardiomyopathy.

So our conclusions are that alone the alpha 2C

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receptor is a risk factor for heart failure, but alone the beta 1 Arg389 is not. However, together they represent a tenfold risk for heart failure. We think this synergism is consistent both with the transfected cells that I showed you, the gene modified mice that I showed, and this sort of series circuit where you have norepinephrine being released and acting on its receptor.

So how can we put this together with what we're talking about today? As we discussed -- and we could spend a long time talking about which adrenergic receptor is localized on which tissue and what the exact circuit is, but it's clear that they are important. It turns out

that most of them are polymorphic in so-called normal populations, and that means they're also polymorphic in dysautonomic populations. Whether they are causing it or having an effect on it would be up to a clinical study to determine.

We have the opportunity to know that their biologic properties have been characterized in cells in gene-modified mice. So you would have the opportunity at the beginning of your study, if wanted to look, for example, for gene modifiers that sort of direct the phenotype in a familial dysautonomic syndrome, you'd have an idea of how to design the study, gain of function, loss of function, agonist affinity, antagonist affinity,

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response to drugs. All these can be taken into consideration in designing the clinical trial.

And these phenotypes include virtually everything that you can imagine about a receptor, the ligand binding affinities, the coupling of the receptor to the G-protein. I didn't show this but expression of the receptor can be altered by polymorphisms in the promoter region as well as regulation of the receptor over long-term stimulation. So all this has to be taken into account.

So I would propose that we need to consider adrenergic receptor polymorphisms possibly in some sort of combination as potential causes or modifiers of dysautonomic syndromes.

Thank you.

(Applause.)

DR. GWINN-HARDY: Let's take a 15-minute break and come back at 3:30.

DR. GOLDSTEIN: After the break, Italo Biaggioni will give his talk, and then we'll go right into the discussion.

(Recess.)

DR. GOLDSTEIN: I want to announce to you briefly, in terms of the scheduling sequence, after Dr. Biaggioni's talk, because of time constraints, we will

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start with questions for Dr. Liggett. He has to leave a bit early. And then we'll continue with more general questions after that.

Dr. Italo Biaggioni is a professor of medicine and pharmacology at Vanderbilt, and he'll be talking about hypertension in autonomic disorders: genetic factors and phenotyping.

DR. BIAGGIONI: Thanks, David. We're running a little bit behind schedule. I'll try to be brief.

I don't need to introduce the autonomic nervous system to this group, but just to simplify things -- and this is a very simplified scheme. So don't write letters if you find anything that is not correct.

There's an afferent portion of it that senses what blood pressure and other many things are doing in the body second by second, central sites where all this information is integrated, and then efferent sites where

sympathetic tone is actually delivered to the rest of the body. So problems in any one of these sections can lead to autonomic disorders, and let me focus here on the ones that will produce high blood pressure.

The obvious one is if you have an alteration in the afferents, you would lose your ability to buffer all the changes in blood pressure and you actually have labile high blood pressure.

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The importance of this receptor has been known for a number of years, and in humans they became clear in this study done in the '50s which I doubt that we will see any such clinical research anymore. But this group of investigators actually -- they used themselves as normal subjects, and when they blocked afferents by injecting lidocaine I believe in the neck on themselves, they saw that blood pressure increased by 20 millimeters of mercury if they did one site. Not happy with that, they did both and then blood pressure increased over 200 systolic, and heart rate increased. Again, not happy with that, they studied patients with high blood pressure, and as you can see, they get huge increases in blood pressure. And they studied then patients with severe hypertension and, you know, I don't think our instruments would record those blood pressures anymore.

So, obviously, these are very important baroreceptors that inhibit sympathetic tone, and there are patients who have damage of this afferent usually as a consequence of surgery. The most common of them, of course, neck cancer and paragangliomas, are transmitted in families. And the characteristic is severe labile hypertension together with increases in heart rate and blood pressure and plasma norepinephrine. Those are triggered by stress, pain, and mainly because they have

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absence of this baroreflex modulation.

This is just one example of a patient in this case with a cold pressor test. We tend not to do those anymore. But even if you start talking to them or if you turn on the lights, you will see this huge increase in blood pressure. This patient was originally described by David Robertson. So very minor stimuli that we could handle all the time in these patients will produce a hypertensive crisis not unlike what Felicia has described in the familial dysautonomia patients.

Well, these afferents actually have their first in the synapse in the NTS, and the NTS actually inhibits sympathetic tone. So it provides inhibitory input to the RVLM where pacemaker neurons are believed to be located. And for many years also, people have lesioned the NTS in animals to produce a very similar clinical picture to the one I described.

You know, we were unlucky to see a patient with a similar problem with an NTS lesion. Of course, the problem then is the cortical stimuli, the stress and everything will drive hypertension. And this is unfortunate pathology of this patient showing two holes

actually where the NTS should be. This is most commonly a familial disorder. In this case it was not, but it shows you the importance of the NTS in inhibiting sympathetic tone. Again, in this patient, handgrip or cold pressor test produces profound increases in blood pressure, and there was complete absence of baroreflex mediated heart rate changes.

David already mentioned the other part of the autonomic problems, that is the central and efferent problems, multiple system atrophy. There are lesions in the brainstem nuclei, in the basal ganglia, and also in the pathways that control sympathetic tone. We believe, for reasons that I'm going to explain later, that the actual pacemaker neurons are intact, the neurons on the RVLM. Unfortunately, we don't have still very good neuropathology of that, but we believe that to be the case. But still, they are not able to regulate sympathetic tone, and every time they stand up, they have orthostatic hypotension.

There's also another group of patients with pure autonomic failure who actually have central problems, but clinically they don't. Perhaps in later stages they do, but clinically we tend to believe that they have no central neurological problems, but clearly they have loss of peripheral nerve and again they have orthostatic hypotension, their main symptom. And that really dominates the clinical picture of these patients. They don't have as much GI side effects, for example, but they have a lot of orthostatic hypotension. That's the reason why they come to us.

And that is shown here. That's normals, systolic/diastolic blood pressure and normal response to posture. And these are patients with pure autonomic failure or multiple system atrophy, and you can see this profound orthostatic hypotension.

Like David showed, norepinephrine is very low in pure autonomic failure patients. They do not respond very well. They respond very slightly to upright posture, but that's due to decreased clearance and not really to increased production. Patients with MSA have near normal plasma norepinephrine, but fail to respond appropriately on standing.

Now, the other thing I would like to point out is even though orthostatic hypotension predominates clinically, as you can see, these patients actually have supine high blood pressure, not unlike, again, the patients with familial dysautonomia.

I just will show you the blood pressure distribution in these patients. This is a number of patients we've collected throughout the years. Supine systolic blood pressure, diastolic blood pressure. So if you define hypertension as blood pressures above 150 over 90, about half of the patients have supine hypertension.

We've been interested in dissecting why patients

without a functioning autonomic nervous system might have that supine hypertension. To make a long story short -- and David has already mentioned some of that -- we believe that in multiple system atrophy, the lesion is proximal to the pacemaker neurons that actually produce sympathetic tone. However, they cannot modulate it. So even though the sympathetic tone is there, it's working on idle, if you wish. They cannot rev it up. So the evidence for that has been the normal or only mildly decreased plasma norepinephrine, and again, David's studies elegantly show that the sympathetic nerves in the heart were able to uptake catechols.

So we decided to test the hypothesis that the supine hypertension was also due to that leftover sympathetic tone, and that will be the case in MSA but not in pure autonomic failure which we believe is actually distal to the origin of sympathetic tone.

To test that hypothesis, we used a ganglionic blockade with trimethaphan to interrupt any remaining sympathetic tone they may have. So again, our bias is, our hypothesis is that the pacemaker neurons that produce sympathetic tone -- and by the way, they may not be in the RVLM. They might be in the spinal cord as well. Those are intact. These patients have residual sympathetic

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tone, but there's an interruption on the pathways that control that, so they cannot control that. If we block ganglionic transmission with trimethaphan, any residual sympathetic tone will go away and we'll be able to see if their hypertension is due to that.

But in patients with pure autonomic failure whose lesion is distal to the origin of sympathetic tone, then there's nothing to block and nothing much will happen in them.

Indeed, again to make a long story short, that's what happened with trimethaphan at very, very tiny doses. In patients with multiple system atrophy, they had a profound drop in blood pressure, almost 70 millimeters of mercury on average. So that means that in some patients it was more like 100. And in patients with pure autonomic failure, they did have some decrease in blood pressure, but not nearly as much as patients with multiple system atrophy.

And this is one example, a patient with Shy-Drager who started with a systolic of 200 and with tiny doses that would do almost nothing to a normal subject, he has a profound decrease in blood pressure with now a systolic of below 100. This study was done with Jens Jordan who used to be here, but probably had better things to do than hear my talk.

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(Laughter.)

DR. BIAGGIONI: So I think these and other data, together with David and other labs, do show that in multiple system atrophy, the hypertension is centrally mediated and is due to residual sympathetic tone, most likely acting on denervated hypersensitive receptors,

although that may be questionable, and unrestrained by the absence of the baroreflex.

In pure autonomic failure, it's still an open question as to what's driving in their hypertension, and whether familial dysautonomia will fit under one or the other I think is not completely known. In pure autonomic failure, the question is more complicated and we don't know yet.

Here we're comparing normal with pure autonomic failure patients without hypertension and pure autonomic failure patients with hypertension. And as you can see, both have profound orthostatic hypotension. Norepinephrine is equally impaired in both. You can argue that it's marginally worse in the ones with hypertension. And renin is, if anything, worse in those with hypertension. So we don't believe it's renin. We don't believe it's residual sympathetic tone. _____ is the same. Does that mean that peripheral resistance is increased?

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So the challenge here is to find what is driving that increased vascular resistance. We know that it is not renin. We know that it is not norepinephrine. We're studying other factors.

But back to our point, what lessons can we derive from these patients? I believe that these are models of sympathetically mediated hypertension, and the fact that sympathetic tone can drive sustained hypertension I think is remarkable. In the field of hypertension, we've been focusing on the kidney and for very good reasons. But this just shows you that sympathetic tone can actually also drive sustained hypertension, whereas in pure autonomic failure patients, it's a model of completely independent hypertension of sympathetic tone.

Now, in essential hypertension, of course, there is a lot of evidence that there is some genetic component of that, and the monogenic hypertension has been described. Most of those have been due to kidney problems, but in essential hypertension I think there's a lot of evidence that abnormalities in sympathetic tone are segregated in a familial way. For example, in African Americans, they responded in an exaggerated way to sympathetic stress. If you do cold pressor tests, there's a greater increase in sympathetic activation with those

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stimuli, and that's transmitted in families.

We know that hypertension goes along with impaired baroreflex function. There's nothing new about that. But even normotensive siblings or children with hypertensive parents have already impaired baroreflex function, and that's also transmitted in a familial pattern.

I will not talk about polymorphism. Stephen, I'm sorry. This is a slide made, about a year old. Every time you make a slide, it becomes obsolete.

(Laughter.)

DR. BIAGGIONI: This is a very rapidly changing field. And I agree completely with you that a lot of the studies of associations have been done without much thought of the cellular phenotype of these. So there's a lot to learn in this field I'm afraid.

But the bottom line is that in essential hypertension, there is a component of genetic autonomic function, and this is a field that is going to advance very quickly. At the same time, I'm afraid that the phenotyping we do in patients is not advancing at such a great a degree. A lot of the problems we have in these association studies is that they take all new-comers, essential hypertensives that have nothing to do with autonomic problems and essential hypertensives that may

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have a greater autonomic component and not an easy way to segregate between both of them.

So how do you phenotype for sympathetic function? The traditional way has been plasma norepinephrine, a very useful marker but not a very sensitive one, and David has run very good reviews or meta-analysis on that. Norepinephrine spillover is a much refined technique, but again very difficult to implement on a population basis.

The same is true of microneuropathy. Very important, very powerful, but again difficult to do on a population basis.

So we've been looking at spectral analysis of blood pressure fluctuations as an alternative to do this phenotyping. And I'm not here to try to sell that as the best technique, because clearly we have to do more work on that.

But just to say a word in terms of spectral analysis, I think most people will agree that high frequency variability of heart rate is an indicator of parasympathetic modulation of heart rate. But there's a little bit more controversy about low frequency variability of blood pressure, and I believe that it has limitations, but I'll show you some data showing that perhaps it is a measurement of sympathetic modulation of

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vasomotor tone.

So normally blood pressure fluctuates in a rhythmic pattern, and you can analyze that in spectral analysis. And it actually goes along with sympathetic nerve traffic measured here with MSNA, and sympathetic nerve traffic is also not continuous. It comes in bursts. That tracks nicely with variability in blood pressure with a lifetime, of course.

Now, if you stand up and you increase sympathetic tone, this variability becomes more pronounced, as well as the burst of sympathetic activation. So you can analyze either or both with spectral analysis techniques.

So we decided to put that as a test. Again, we're using our ganglionic blocker, the idea being that if low frequency oscillations of blood pressure are a marker

of sympathetic tone, if you wipe out sympathetic tone, you will wipe out oscillations of blood pressure.

Again, we used as our positive control patients with multiple system atrophy, which we've already shown they are sympathetically mediated, and as a negative control, patients with pure autonomic failure, patients which again don't have much sympathetic tone left.

I just will show you that trimethaphan is actually very effective in wiping out the autonomic

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function. Here we're looking at baroreflex sensitivity, and we're looking at patients with pure autonomic failure who already have almost no baroreflex sensitivity. Patients with Shy-Drager, multiple system atrophy, also have very low levels. So in them trimethaphan will do not much. Normotensives have a normal baroreflex function, and hypertensives have been shown over and over again to have impaired baroreflex function, and that's shown here. With trimethaphan, you can also wipe that out. So, it's a very effective way of eliminating any remaining autonomic tone.

Let's concentrate on low frequency oscillations of blood pressure. Here we see pure autonomic failure patients within the noise level. So they have very low oscillations, and this also shows you that the limitation of this technique is that it's very hard to show a decrease in sympathetic function using this technique because levels lower than 1.52 are really noise. But if you have normal subjects, shown here in the circles, you can also reduce that with this drug, with a ganglionic blockade.

Now, in patients with multiple system atrophy, who have profound orthostatic hypotension but we've been postulating have residual sympathetic tone, they have actually increased oscillation, together with patients

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with hypertension. So these are patients with supine hypertension and Shy-Drager and patients with essential hypertension, and both have increased oscillations in blood pressure and both can be abolished by a ganglionic blockade.

Just to show you that this is not a fluke, high frequency oscillations of blood pressure, we believe, are many less, and indeed if you use trimethaphan, they go nowhere. So again there's a nice internal control that what we're seeing here is actually physiologic.

And that goes along with the findings in terms of blood pressure. So if sympathetic tone is driving your blood pressure, you wipe out sympathetic tone, you should lower blood pressure. And that's what we see in Shy-Drager patients. In normals, there's some drop in blood pressure, as you would expect. Some of the even supine blood pressure is maintained by blood pressure, not a lot, but some of it. Of course, if you stand up, then they will fall, faint. But supine blood pressure is not driven a lot by sympathetic tone. So wiping out sympathetic tone lowers blood pressure a little bit in normotensives, as

well as in patients with pure autonomic failure. But in Shy-Drager, again a model of sympathetically driven hypertension, even with tiny doses, you have this profound, even 150 millimeters of mercury, drop in blood

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pressure.

In essential hypertension, it's a mixed bag. It was actually what we had predicted that essential hypertension is not one disease. It's probably many, and they may have different components of autonomic tone. And in some patients, we were not able to give greater doses because their blood pressure dropped. In others, they were able to withstand even higher doses of trimethaphan.

So at the end, once we blocked autonomic tone, we come up with an intrinsic blood pressure, that is, what is your final blood pressure in the absence of autonomic tone. And if you're normal, that's usually below 125, 120. If you have Shy-Drager, all except one fall below that level. And again, these are patients that started with very, very high blood pressures.

In pure autonomic failure patients, they remain hypertensive, meaning that their hypertension is not driven by the sympathetic nervous system.

And in hypertensives, it's a mixed bag. Some will have sympathetically independent hypertension and some will be sensitive to sympathetic blockade. And I think that may be a way, although a lot more work -- and we need to study more patients, of course -- needs to be done. I think that may be a way of phenotyping which subset of patients with hypertension will have greater

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sympathetic component and those will be the ones we might focus on in terms of genetic testing.

So to conclude, autonomic failure patients provide us with unique models of sympathetically driven and sympathetically independent hypertension. Trimethaphan especially and perhaps low frequency variability of blood pressure might be useful in phenotyping the contribution of sympathetic tone to hypertension. And let me also add that trimethaphan we find also useful to dissect patients who have a central autonomic problem to those who have a more distal autonomic problem. And again, that might also be helpful in familial dysautonomia.

I think I'll stop here and hopefully we're caught up.

(Applause.)

DR. GOLDSTEIN: The initial questions should be directed to Steve. So, all the speakers from my session. Is anybody still here?

DR. BIAGGIONI: I think Steve can probably answer all the questions you might have.

(Laughter.)

DR. GOLDSTEIN: You're leaving Steve Liggett off the hook.

DR. EISENHOFER: No, I'm not. I'm Groeme

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Eisenhofer from the NIH.

Steve, I wonder if you've done any studies to -- I thought they were elegant associations with heart failure, but in terms of the phenotyping, actually a little bit deeper in looking at the sympathetic nervous system in these patients with the alpha 2 polymorphisms in particular.

DR. LIGGETT: What I'd really like to do is measure norepinephrine turnover in the cardiac sympathetic nerve system in the cleft and that can be done. It's fairly complicated and we're sort of about halfway there.

DR. EISENHOFER: Well, maybe we might be able to help you with that.

DR. LIGGETT: Okay, that will be fine.

DR. EISENHOFER: We have a lot of experience in those. Dave Goldstein and myself.

DR. LIGGETT: Right, but I think that's the way these studies probably are going to go. They're going to be small physiologic studies, big association studies, animal studies, and cell studies, and when you put the whole picture together, you're convinced of what's going on. But any one study doesn't quite do it for me.

DR. EISENHOFER: I agree.

DR. GOLDSTEIN: It would be especially worthwhile to find out what happens to the spillover of

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norepinephrine in the heart in response to a drug like yohimbe, for instance, which blocks those alpha 2 receptors and is known to increase norepinephrine spillover in the heart.

Of course, the interaction doesn't have to be just among polymorphisms for adrenoceptors. There's I think a very high likelihood that polymorphisms related to the transporter and polymorphisms related to alpha 2 adrenoceptor function will interact because in people who have a pharmacologic blockade of the transporter, it drastically increases their norepinephrine spillover and blood pressure in response to an alpha 2 blocker.

DR. BROWNSTEIN: I wonder if any or all of you could comment on the relationship between long QT interval and autonomic imbalance.

DR. BIAGGIONI: The QT?

DR. BROWNSTEIN: Long QT.

DR. BIAGGIONI: Yes. We see QT prolongation in patients with autonomic failure, and we can induce it with ganglionic blockade as well. But it's hard to know whether these patients have an increased risk of arrhythmias, for example, because of that because we don't follow them in a very systematic way. Some of them have a very poor prognosis. So it's not clear the function or the relevance of that.

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DR. GOLDSTEIN: I don't think anybody knows.

DR. BROWNSTEIN: It's relevant, of course, to FD because those patients also have long QT. They probably get in trouble because of it, and the issue for me is whether or not this is primary, in other words, whether or

not the splicing defect could injure myocardial function directly or whether it's secondary to a dysautonomia. So if you could comment on that based on your experience with other --

DR. GOLDSTEIN: Max, why don't you respond?

DR. HILZ: I'd just like to mention that there was a paper in '91 or '92 on comparison of QTc in Romano-Ward patients and MIBG uptake showing that the reduction of the cardiac MIBG uptake is even a more sensitive marker for Romano-Ward patients than the patients than the QTc prolongation. So obviously there's evidence of structural dysfunction at the level of the myocardial sympathetic innervation.

DR. GOLDSTEIN: Yes. There's a guy named Peter Schwartz I think in Italy who has tried to sell the idea that there are patients who have prolonged QT and susceptibility to sudden death related to an imbalance between the left and right sympathetics coming the stellate ganglion. I think he's actually carried out a large number of left stellate ganglionectomies in an

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attempt to treat these people. He's not here, so I can say whatever I want.

(Laughter.)

DR. BIAGGIONI: I think that the patients with FD might be more at risk for QT prolongation because of their crisis. So in our patients with autonomic failure, they have very low sympathetic tone or nonreactive sympathetic tone all of the time, so they have QT prolongation, but normally they will do fine. Now, if we give a beta agonist, they will have a lot of arrhythmias.

Now, in the patients with familial dysautonomia, during those autonomic crises, they might be particularly at risk, and I wonder if you have seen that, Felicia, if you have seen arrhythmias during those crises.

DR. AXELROD: We haven't seen arrhythmias during crisis except actually during the retching, and at that time they develop often a bradycardia. And the QTc has not been as sensitive as the JTc because the QTc takes care of polarization and depolarization, so it's too much; whereas, the JTc is just repolarization which is more under sympathetic control. So I think it's been a better predictor.

And actually we did a study where that subpopulation that had JTc prolongation actually had increased problems with syncope and some of them succumbed

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to -- we did it prospectively -- sudden death.

DR. GOLDSTEIN: It could also be that there's heterogeneity of sympathetic innervation within the myocardium that could result in kind of a dispersion of repolarization, and as a result, since you're only looking at the end of the T wave, all you can say is what the maximum duration of repolarization is. But there could be a lot of heterogeneity in the timing of the repolarization and that could itself be a risk factor.

DR. AXELROD: Yes, that could but that's again

modifying genes and things to think about. But JTc prolongation might be one indication for use of pacemaker in these patients.

DR. GOLDSTEIN: Other questions for Steve Liggett?

QUESTION: This is a selfish question, but per all your work, do you see any application for familial dysautonomia and has Vanderbilt actually worked on familial dysautonomia in the past since I know they do work on all the dysautonomias.

DR. BIAGGIONI: We saw no reason to work on familial dysautonomia because Felicia had already taken the whole field.

(Laughter.)

DR. BIAGGIONI: No. We don't see children

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really, so we haven't been as active.

QUESTION: And how about the other work? Does any of the other work have an application, do you think, to familial dysautonomia?

DR. LIGGETT: Well, I think as a general concept we might even think of cystic fibrosis in a similar manner to what we see here. So the delta 508 mutation that causes 70 percent of cystic fibrosis is in the CFTR gene. Yet, if you look at all the patients who are homozygous for that mutation, the variability in clinical presentation and various aspects of the disease is quite broad from those who just have lung disease, those who have more pancreatic problems, et cetera. And for those individuals, just like your patients, their life span has improved but it's primarily due to supportive care and understanding nutrition and various aspects. But they've not made a lot of progress in moving beyond that.

And I think that one of the hot areas in CF is trying to figure out what modifier genes are controlling why some people have a predominance in one organ system versus another. Since this is an autonomic conference, I got to think that autonomic receptor variation, which is normally tolerated fairly well in people, but if you have this bad disease plus an altered autonomic receptor system, it could give you a predominance of one symptom

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versus another.

So I think it is actually potentially applicable. It just needs to be done with the right populations and the right phenotyping in the patients. I guess you would probably want sort of extreme phenotypes, those with no GI disorder or those with one phenotype or the other so that you get a maximal chance of finding something.

QUESTION: You're right. Most of the work done on FD is with Felicia Axelrod. I do think there might be an advantage to get other points of view and other people working on it. Just as with Mass General working on the gene, they later on saw that the way they grow their samples had a big impact. And I think they were only able to see that once another group had come to a finding as

well. So I think there's always room for extra people.
DR. BIAGGIONI: No, and we actually do that. We've done that in the past. I think Felicia has worked with us on other things, with David, and with Max, with a lot of people in the field.
DR. GOLDSTEIN: I have an open invitation. Any patient over the age of 18 with FD I'd be very interested in finding out whether the sympathetic innervation of the heart really is there or not. I think it's very doable and I'd be happy to do it.

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DR. AXELROD: Well, I think that's really a very important point. I want to address two things.
One about modified genes. I think as Dr. Peltzer eloquently described, even within the same family, you have two children who have very, very different kinds of courses, and other families where there has been more than one child affected, the same kind of thing has happened where you don't exactly see the same disease expression, but there's modification within there. So we have to assume that there are some modifying genes, and I think Dr. Hirschhorn also tried to alert us to this possibility. And the search for modifying genes is probably going to help us explain disease variability.
I think collaborative studies are really important. What I said in my introduction, this hopefully will be the fruit of this kind of conference, and I think this is the spirit of this kind of a conference.
Dr. Goldstein has been wonderful. He actually has studied one of our patients, the sympathetic innervation of the heart in one patient already, and we hope that other patients will cooperate. When they had trepidations, I came down to the NIH and held the hand of the patient so they wouldn't be as frightened of Dr. Goldstein.
(Laughter.)

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DR. AXELROD: Yes, a very scary man.
(Laughter.)
DR. AXELROD: No, but I think if Vanderbilt has some good ideas and other institutions have some good ideas, that would help patients, I don't want people to think that I will not cooperate or encourage my patients or even just stand by to hold their hands.
DR. PELTZER: There's one interesting thing with the variation. There is one case of identical twins that both have FD, and they actually have a different presentation. They are not identical in their presentation. So absolutely looking for other genetic factors is important, but there clearly are some environmental factors that are impacting on the disease as well.
DR. GOLDSTEIN: We'll open it up to questions on any topic from this session.
I have a question for Italo. If in PAF there's a denervation and yet the patients have significant supine hypertension, other than the low baroreflex sensitivity,

what else comes to mind about why that would be?
DR. BIAGGIONI: I think we're just starting to try to find out. So when you look at what maintains blood pressure or what maintains blood pressure under a relatively narrow limit, obviously the autonomic nervous

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system is important, renin is important, and there are several other things. These patients have taught us that nitric oxide is also very, very important. So this is a compound that is produced by the cells lining blood vessels. In normal subjects, if you block that, you increase blood pressure by 10, 15, 20 points. But that is misleading because that is a normal subject with a normal autonomic nervous system. So every time you try to increase blood pressure, the autonomic nervous system will try to reduce it. If you block autonomic function, then nitric oxide, then blocking nitric oxide increases blood pressure by 30, 40, or 50 points. So this is obviously one good candidate that we're looking at, but we're just starting those studies.

DR. GOLDSTEIN: Yes, I was wondering. I was thinking along the same lines, but also in terms of treatment, if there were a way rapidly to interfere with nitric oxide synthase, then given the possibility that the PAF patients have supine hypertension because of some endothelial dysfunction, let's say, maybe you can exploit that and allow their blood pressure to increase by basically blocking that nitric oxide synthase temporarily when they want to be up.

DR. BIAGGIONI: Right.

DR. ADLER: Suzanne Adler from Melbourne,

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Australia.

A question for Dr. Ando. Can you comment on the use of L-threoDOPS in the patients with amyloidotic polyneuropathy?

DR. ANDO: Yes. That was very effective for also static hypotension with elevation of the basal blood pressure. As I said in my presentation, severe diarrhea simultaneously occurs usually. So that chemical compound was effective for treating diarrhea partially.

DR. GOLDSTEIN: For those who don't know, L-threoDOPS is one of the four stereoisomers of DOPS. DOPS, dihydroxyphenylserine, basically is an amino acid, looks exactly like DOPA, except just as DOPA gets turned into dopamine, L-DOPS gets turned into norepinephrine. So, L-DOPS is being used as a kind of a norepinephrine prodrug in several forms of autonomic failure and is virtually curative in dopamine betahydroxylase deficiency where this is essentially reversing the defect.

DR. ANDO: Yes. The very useful artificial precursor compound of norepinephrine is converted in the plasma in 3 or 4 hours after administration. However, in FAP patients, severe diarrhea with sometimes tablet in the stool. So we made a nasal application for FAP patients.

DR. ADLER: What's the time of onset of action given intranasally?

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DR. ANDO: Time of what?

DR. ADLER: How quickly does it work given intranasally versus orally?

DR. ANDO: Eight times a day or so makes sense for FAP patients' therapy.

DR. ADLER: And a question to you and also perhaps to Dr. Biaggioni. Do you think there's a role for a trial of L-threoDOPS in familial dysautonomia related postural hypotension?

DR. GOLDSTEIN: Oh, yes, if you can get the drug.

DR. ALDER: If you can get the drug. If one is persistent, one might. Do you think it's worth a try?

DR. BIAGGIONI: Well, I think, Dr. Kaufmann, perhaps you might want to elaborate a little bit on that. You have some experience with using that drug in other patients with autonomic failure.

DR. KAUFMANN: I don't have a lot to say. David already summarized it. This is a very interesting drug. It's a synthetic precursor of norepinephrine. And the first group that used it was David and Italo and another group in Europe for patients with DBH deficiency, with dopamine beta-hydroxylase, which David is going to talk about tomorrow.

We were lucky enough to have the compound and we

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still use it. So we did a trial together also with David and with Roy Freeman from Boston in patients with multiple system atrophy and with pure autonomic failure, and it was dramatically effective. It was very, very good.

So in familial dysautonomia, I cannot tell you. I have very little, the same as was said before. All the patients with familial dysautonomia I have seen were when I went doing rounds at NYU with Felicia, and a few that were upset with Felicia that came to Sinai.

(Laughter.)

DR. KAUFMANN: And I understood why they were upset with Felicia because anybody would be upset with them. They were very difficult patients. So I think it would be interesting to try.

DR. GOLDSTEIN: Felicia, have you ever given the stuff to an FD patient?

DR. AXELROD: (Inaudible.)

DR. BIAGGIONI: When we used it, it was not commercially available, and I think we bought it through Sigma Chemical Company for oral use, and it was like $50 a dose. So without support from a drug company, it would be very difficult to do.

DR. KAUFMANN: (Inaudible.)

DR. BIAGGIONI: Right, it's not as effective. So it will be $100 per dose.

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DR. STEWART: Julian Stewart, Valhalla, New York, for Dr. Biaggioni.

A comment about blood pressure variability. In my experience, whenever heart rate variability became

essentially monotonous, blood pressure variability went haywire. My thought was -- and perhaps you can answer whether it's your thought as well -- is that on a beat-to-beat basis, the heart rate may serve a role in compensating for wide swings in blood pressure. What do you think?

DR. BIAGGIONI: Yes, of course, right.

DR. STEWART: That's it.

DR. BIAGGIONI: Yes.

(Laughter.)

DR. STEWART: So that when you have no baroreflex transfer, for example, you're going to see wide swings in blood pressure generally speaking.

DR. BIAGGIONI: Right.

DR. STEWART: As you do in baroreflex failure.

DR. BIAGGIONI: Right.

DR. GOLDSTEIN: I think what he's trying to say is that the issue of sympathetic regulation being measured by the effects of trimethaphan on the power spectral analysis of systolic pressure may be influenced or even determined by a concurrent baroreflex regulation of pulse

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rate, and trimethaphan is eliminating both parasympathetic and sympathetic postganglionic traffic.

DR. STEWART: I see the same thing in patients with changes in baroreflex gain so that as the gain falls down, the blood pressure variability -- as the heart rate becomes monotonous, blood pressure variability almost invariably skyrockets, for whatever reason.

DR. BIAGGIONI: Can I ask a question to Dr. Ando? Do your patients have supine hypertension?

DR. ANDO: Basically no, but after administration of L-threoDOPS _____, supine hypertension sometimes occurs. Basically no.

QUESTION: A couple of years ago, I did ask someone at the center -- I forgot who -- on FD Day about L-DOPS because I read such great stuff about it, but at the time I think someone didn't hear about it and someone did, but they were afraid that when the blood pressure in FDers go up during a crisis, that the L-DOPS might push it over the top and that might be a problem.

DR. BIAGGIONI: I guess we need to understand better why they have these crises. Well, let me rephrase that. I would need to understand better. Perhaps somebody knows. But if it is driven by the dopamine or epinephrine or dopamine being converted to norepinephrine, you're going to be competing with the same receptors. So

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I'm not sure if it's going to make it worse.

DR. GOLDSTEIN: I think a lot depends on whether there's denervation. That's crucial because if there is denervation, then you could argue that with a crisis, the L-DOPS isn't really going to make that much of a difference, but if there is innervation, then the L-DOPS would be converted to norepinephrine. Norepinephrine could be taken up into those existing terminals, and the norepinephrine could be released during sympathetic

stimulation. And that would be dangerous. So I think a lot depends on whether there's really denervation or not.

I wanted to mention -- remember back when I was talking about the five components of the autonomic nervous system -- I think it's really crucial to find out if during these crises there actually is a high plasma epinephrine level because it sounds to me like a lot of the findings could be explained by a combination of epinephrine and receptors that are super-sensitive.

DR. AXELROD: There isn't. That's been done. There's high dopamine during crisis and some patients actually have some high norepinephrine, which we believe is converted from the dopamine because it's seen with longer crises. When the crises last a long time, then you see a lot of hyperglycemia, hypertension, you see the norepinephrine go up, but the epinephrine is not up.

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DR. GOLDSTEIN: I've been confused by that dopamine story because I know if you infuse dopamine into normal volunteers to produce these levels of dopamine -- granted, they're 10 times normal -- there's no hemodynamic effect. In order to see an increase in blood pressure with infused dopamine, you have to have a concentration in a range of about 3,000 picograms per ml. That doesn't happen, not even during a crisis.

DR. AXELROD: (Inaudible.)

DR. MAAYAN: I'm just saying that for the same amount of dopamine, because receptors of dysautonomic patients are hypersensitive, they're reacting much more.

DR. AXELROD: Actually that was also done by Weiser and myself in a microcirculation study with the laser Doppler, and we actually demonstrated hypersensitivity of the adrenoreceptors. So they could bind to --

DR. GOLDSTEIN: To dopamine?

DR. AXELROD: -- other catecholamines. I mean, the dopamine can bind to that as well.

DR. GOLDSTEIN: Did you find that there was an effect of dopamine on the --

DR. AXELROD: We did not use dopamine.

DR. GOLDSTEIN: -- flowmetry?

DR. AXELROD: No, we didn't use dopamine during

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the microcirculation studies. We used norepinephrine.

DR. GOLDSTEIN: Right. The adrenoreceptors I'm sure could be up-regulated, but the issue is what dopamine is doing.

Yes, go ahead.

DR. ADLER: The crisis is such an awful part of dysautonomia. Given that we have more technology now to look at catecholamine chemistry and dynamics, it might be worthwhile devising a protocol where we actually look at what happens during crisis using all modalities in a monitored lab, because we really are in the dark knowing what drugs to blocks, to treat when we go into crisis. The drugs we use for day-to-day staying upright and not falling dizzy are the opposite to what you might want to

have around when you're having crisis. So we need to look at short-acting agents, we need to look at alternative routes of delivery, sublingual, intranasal, whatever. Parents are capable. They can even given subcutaneous injections if there was a drug that was appropriate. So I think it's time to probably work out another protocol for a study for crisis, and I'm sure we could find lots of volunteers.

DR. GOLDSTEIN: Thank you.

All right. I think we'll call it a day. Thanks everybody.

0236

(Applause.)

(Whereupon, at 4:25 p.m., the meeting was recessed, to reconvene at 8:30 a.m., Friday, October 4, 2002.)