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International AIDS Society
2nd IAS Conference on HIV Pathogenesis and Treatment
Main Plenary – Mechanisms and Management of Metabolic Complications Associated With Highly Active Antiretroviral Therapy
July 16, 2003

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JEAN-FRANCOIS DELFRAISSY: Good morning. My name is Jean-Francois Delfraissy. I'm Co-Chair of the conference. This morning we have two topics, the one on HIV entry and the second on complication. It's a pleasure for me to introduce Bob Doms. Bob obtained his Ph.D. and M.D. at the Yale University, and then he moved to (unintelligible), and recently joined the faculty of the University of Pennsylvania, where he is now an Associate Professor of the Department of Microbiology. His lab was one of those that discovered the (unintelligible) coreceptor, and with 32 CCFI (unintelligible). And his recent work has (unintelligible) and is in addition with (unintelligible) drugs. His work has been recognized by (unintelligible) from the (unintelligible) Gates Foundation and by Pfizer investigate (unintelligible) from the American Society for Investigative (unintelligible) Project. Bob.

DR. ROBERT DOMS: Thank you, and I'd like to thank the organizers for the opportunity to talk to you this morning about how HIV enters cells, how HIV entry can be inhibited by a new class of antiretroviral agents that are under clinical development and some of the challenges that we will likely face as these new drugs move through clinical development. So, to understand how entry inhibitors, you have to know something about how viruses surrounded by lipid envelopes like HIV get into cells. So in order for HIV or any envelope virus to

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infect a cell, it has to introduce a (unintelligible) from inside the virus, inside the cytoplasm of the host cell. In order to accomplish this, the virus faces two physical barriers, the barriers that are imposed by the membrane of the virus and the membrane of the cell. And since biological membranes are stable structures, this represents a significant biophysical barrier. So all envelope viruses, regardless of whether you're talking about SARS or influenza, rabies, or HIV, overcome these physical barriers by including at least one viral membrane protein, that under the right conditions, changes it structure in such a way that it will (unintelligible) between the membrane of the virus and the membrane of the cell, and as a result, the viral genome gains entry to the cytoplasm. So, regardless of the type of envelope virus you're talking about, the fundamental steps of virus entry are the same. The first step is simple attachment of the virus to the cell surface, which may or may not involve specific receptors. In the case of HIV, that would be CD4. So now the virus is attached to the surface of the cell. The next step is there has to be a trigger event. There has to be some way to impart information to the viral envelope protein that it's time for them to go to the conformational changes that lead to membrane fusion. And there are basically two types of triggers. So viruses like West Nile virus, like influenza virus, the trigger's actually acid pH. After binding to the

cell's surface, the virus sort of sits there like a Trojan horse and is eventually (unintelligible) and delivered to endosomes, which are acidic vacuoles within the cell, and the protons, within the endosome, actually serves the trigger. Acidic residue, they are proteinic, that breaks salt bridges, that enables the viral protein to undergo the conformational changes that lead to fusion between the membrane of the virus and the membrane of the endosome. So you have a trigger event---in this case, acid pH--leading to a conformational change, which leads to membrane fusion. HIV basically does the same thing, but it uses a different triggering mechanism. HIV is a pH independent virus. It does not require acid pH to induce the structural alterations that lead to membrane fusion. So as a consequence, it has the potential, at least, of fusing directly with the surface of the cell. It still needs a trigger event, however. An HIV entry is triggered by sequential receptor engagement. So for all primary HIV-1 stain, the first critical step is binding of the viral envelope protein for CD4, which is, as you know, expressed on certain classes of T cells and macrophages and (unintelligible) cells. The CD4 is just the first step. CD4 alone is not sufficient to trigger the changes that lead to membrane fusion. For that to occur, the viral envelope protein has to engage a second receptor termed the coreceptor, and it is that binding of that, shown as step 2 here, that is widely thought to be the trigger

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that leads to the changes that ultimately lead to fusion between the membrane of the virus and the membrane of the cell. And we now know that there are 2 important HIV coreceptors: the chemokine receptors, CCR5 and CXCR4. A number of other (unintelligible) trans membranes and main receptors can also support virus entry in vitro when they're expressed at very high levels, but there's really no compelling evidence that any of those alternative coreceptors are important in humans. When people become infected with HIV, they almost invariably become infected with viruses that use CCR5 as the primary receptor, and some individuals who progress to AIDS continue to harbor viruses that use CCR5. You know, in some individuals—and it's estimated that approximately half of individuals who progress to AIDS, there's what we call a clear receptor switch. As a consequence of mutations that accumulate within the viral envelope protein, the envelope protein now uses a related receptor called CXCR4, either in place of CCR5 or in addition to it. And while you don't have to have a clear receptor switch to progress to AIDS, if viruses that use CXCR4, so-called X4 viruses, can be detected in individuals, it's usually a bad prognostic indicator because these viruses are associated with accelerated disease progression, since CXCR4 is expressed in a much larger fraction than CD4 positive T cells than is CCR5, so that the virus undergoes a coreceptor switch to utilize CXCR4, it, in effect, broadens its tropism to a much

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larger fraction of your CD4 positive cells. Now, the real importance of the chemokine receptor for HIV entry was shown in 1996, when it was discovered that some individuals, particularly Caucasians from Northern Europe, lacked CCR5 as a consequence of an inactivating 32-base pair deletion and to open a reading frame for CCR5 gene. The mutative protein, instead of spanning the membrane seven times, traverses the membrane four times, the protein is grossly misfolded, and is not expressed on the surface of the cell. So, as a consequence, individuals, shown on the bottom, who have two copies of this mutant delta 32 allele, are CCR5 knockouts. They don’t have CCR5, but they are otherwise normal. You apparently do not need CCR5 for normal human growth and development. But individuals who lack CCR5 are highly resistant, they’re not absolutely immune to HIV infection. Now, importantly, heterozygotes, individuals with one copy of this mutant allele, have a very slight degree of protection of virus infection, but when infected, on average, progressed to AIDS more slowly, somewhere between 2 and 4 years. And as far as anybody can determine, this is due a relatively modest reduction in the amount of CCR5 present on the surface of their T cells and macrophages. And this is important, it becomes important later, because I think what it says is that CCR5 is rate limiting for virus infection. There is considerable natural variation in CCR5 expression levels between

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individuals, and CCR5 expression is one (unintelligible) factor that impacts the rate of disease progression. So nature has done this incredible experiment that has shown how CCR5 is for HIV and how unimportant it is for human growth and development, and that makes CCR5 then a really good (unintelligible) target. Early last year, the first CCR5 antagonist results from the first clinical trial were reported, and now a number of CCR5 antagonists, small molecules that bind to CCR5 and prevent HIV from using it, are now under clinical development. So (unintelligible) was the first small molecule antagonist, and there are several others that are in patients now, and early trials have shown that this antagonist does, in fact, reduce virus load. The big question in using CCR5 antagonists is that if you apply these to a patient for a prolonged period of time, will this simply select for viruses that will (unintelligible) fuse with CXCR4 coreceptor, which could be an important outcome, given that these viruses are associated with disease progression. And nobody really knows the answer to that question. You can argue either side of the equation. I think there are some very good reasons to argue that such a coreceptor switch will not occur, particularly in patients who are treated relatively early after an infection, but ultimately no one really knows, and we'll have to see what happens at the clinical trials. One of the implications of using coreceptor antagonists as therapeutics, though, is that new clinical tests

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will have to be employed. It will no longer be sufficient to simply measure how much virus is circulating in the patient. It becomes to determine what kind of virus is circulating. If you have a patient that you have predominantly viruses that use CXCR4, it would make no sense to give to that person a CCR5 antagonist and vice versa. So viral phenotyping will have to become an important new clinical test in order to help guide the application of these drugs as they move through development. So how does membrane fusion actually occur and how does the virus get in? So on the side of the virus, there is the envelope protein; on the side of the cell, there is CD4 and a coreceptor. So the HIV envelope protein is a trimer. It three identical nonprevalently (misspelled?) associated subunits, and each subunit, in turn, consists of two parts. There is a surface gp120 subunit, that I'm showing here as the large red ball. This is responsible for binding to the CD4 and a coreceptor. And gp120, in turn, is associated with a membrane scanning subunit, termed gp41. So gp41 scans the viral membrane. So within the trimer, you have three gp120s, you have three gp41s. So, first the envelope protein engages CD4, and the number of CD4 molecules needed to trigger the conformational changes that enable the virus to bind to coreceptor likely varies between different virus strains. Some virus strains may need but one CD4 to trigger a trimer, whereas others may need two or three. CD4 binding triggers at least

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two conformational changes in envelope protein that you can measure. The first results in the exposure of a highly conserved (misspelled?) region in gp120, termed the coreceptor binding site, which I'm showing here as a pink ball. So this (unintelligible) is highly conserved, it's kept hidden, and CD4 binds to gp120. These variable loops, including the third variable loop and the first and second variable loops, sort of swing out of the way, exposing this previously hidden domain that plays an important role in binding to both CCR5 and CXCR4. The second aspect of conformational changes induced by CD4 is that it induces some changes in gp41 that expose the binding site for a new entry inhibitor called T20 or Fusion, which has gotten FDA approval now to be used in the United States and in Europe. So CD4 binding alone apparently makes envelopes susceptible to this fusion inhibitor. And we have a good idea of what these conformational changes look like, because several years ago a core fragment of gp120 was co-crystallized with CD4. And the orientation of this picture, you would be the cell, and the virus was coming towards you, the gp120 is shown as a space-filling model, and pink and red are residues that are highly conserved in part of this coreceptor binding site, and that will bind to either CCR5 or CXCR4, depending on the strain of virus that you have. Okay. So CD4 binding now makes it possible for the envelope protein to bind to its coreceptor, be it CCR5 or CXCR4. And this is probably the trigger, then,

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that leads to the final conformational changes. And it's widely thought that one of the first steps is exposure of what's termed the fusion peptide, which is the very beginning of gp41. It's a stretch of hydrophobic residues that are thought to physically insert into the membrane of the cells or to stab the cells. So, as a result, gp41 now becomes an integral component of two membranes, the viral membrane, of which it's normally launched, and the cell membrane, that it's just stabbed. So now you've got the cell membrane and the virus membrane physically tethered by gp41. So how do you actually get the lipids together, how do you cause fusion, and how do you stop it? So you need to look at the process a little bit more carefully. In the top left is what I showed you in the previous slide, which is the envelope that is bound to CD4, bound to coreceptor. The fusion peptide is exposed and inserted into the membrane of the cell. And below that, I just showed the three gp41 subunits in the trimer. For simplicity, I've taken away gp120. If you look at the amino acid sequence of gp41, and depicted schematically shown on the bottom, you see the virus membrane off to the right. To the left is the hydrophobic fusion peptide, that stabs into the membrane of the cell. If you look at the rest of the gp41 sequence, you can see that there are two domains that like to form out the helices--helical regions one and helical regions two. The yellow HR1 domain interacts with the core (unintelligible) HR1

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domains in the other subunits in the trimer, so you get a triple-stranded coiled coil that projects the fusion peptide in the direction of the cell membrane. And then what happens is the molecule basically folds back on itself, helical region two flips back and binds to HR1, sort of like an umbrella. And when this happens, it drives together the transmembrane domain, which is lodged in the virus membrane and the fusion peptide, which is in the membrane of the cell, brings the lipid bilayers into close proximity. And it is likely that somewhere between 3 and 5 trimers of the envelope protein have to undergo this structural alteration at about the same time in order to drive membrane fusion, generate a fusion pour (misspelled?) to enable the virus to enter the cell. So this brings up T20. So T20 or Fusion is a 36-amino-acid-long peptide that you inject it subcutaneously twice a day. And T20 is basically a peptide that's based on the sequence of the HR2 domain, and as such, it does what the HR2 domain normally does--it binds to the HR1 domain, and by doing that, it blocks the formation of the six-helix bundles of envelope, blocks the snapping together of HR1 and HR2, and so it blocks membrane fusion, and now you have a dead virus. So an important point to keep in mind with T20 is that it targets a structural intermediate of the fusion process. T20 cannot bind to the native virus because its binding side is not exposed. Its binding side becomes exposed only after the envelope protein is down to CD4, then there's a

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kinetic window (unintelligible) T20 function. And if it binds to envelope within that kinetic window, it blocks the final conformational changes that lead to membrane fusion. So this movie that's been produced by Trimaris (misspelled?) shows this process in far better detail, I think, than my two-dimensional slide does. This shows the virus zooming in. The surface of the cell is below. CD4 is shown in blue. CD4 has four immunoglobin-like domains, so each CD4 molecule has four blue balls, and the coreceptors are shown as these inconspicuous orange dots. So the envelope protein comes in and binds to CD4. And here it shows three CD4's binding to each of the gp120 subunits, which is true for some virus strains. That induces conformational changes, enabling the envelope to bind to the coreceptor, the fusion peptide becomes exposed and basically stabs the membrane of the cell. So now you have the two membranes tethered, and now the HR2 domains slip back, they form out the helices on these grooves on the outside of the triple string of coiled coil, and basically snaps the membrane together. And when several of these operate in concert, you get a fusion pour (misspelled?). And now the virus is in the cell, and virus replication can proceed. I should say that many other envelope viruses like influenza and Ebolas do exactly the same thing. Their envelope proteins also form these six-helix bundles. This is how T20 works. T20 are these, you know, little bed springs that are floating around,

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and they're floating around because T20 cannot bind to the native (misspelled?) envelope protein because the binding site is not exposed. Again, the virus comes in, and there's good evidence that CD4 binding alone triggers the conformational changes that expose or create binding site for T20. So you've got binding—gp120 is removed from the movie simply for simplicity. There's no good evidence that it actually disassociates. T10 binds, and I suspect you probably are only going to need one binding event per trimer to inactivate that trimer, and now the six-helix bundle formations can't occur, you don't get membrane fusion, and you get a dead virus. So that's how T20 works. So, we have coreceptor antagonists that are under clinical development, that block engagement of coreceptors. You have at least one fusion. There are several fusion inhibitors that one can think about using to block membrane fusion. A challenge that we all face in using entry inhibitors, though, is that they exhibit variable potency, at least in vitro. And there are clearly virus factors and host factors that strongly influence the efficacy of these drugs. And you can show this in two different ways. You can collect different virus strains from throughout the world from patients who have not received entry inhibitors, then infect the cell line, for example, with these viruses, and ask, if I wanted to inhibit the virus replication, how much drug would I have to use? And if you do that, you find that if you used an RT or a

protease inhibitor, depending on the virus strain, these are all from drug naïve patients, maybe you'd have twofold to fourfold variation, not a lot of variation. But when you think of the same viruses and try to inhibit them with T20 or coreceptor antagonists, you easily encounter two orders of magnitude difference in virus sensitivity and sometimes as much as three or four orders of magnitude; so that, some viruses that for some reasons are simply more resistant to these entry inhibitors than others to a considerable degree in in vitro assays. So clearly, there are virus factors that impact entry inhibitor sensitivity to a much greater extent than they do for other antiretroviral drugs, again from drug naïve patients. There are also postcell factors. So in this case, you can take a single virus strain, infect PBMCs from different people, and ask how much drug do I need to inhibit this virus? And what you find is that if you use RT or protease inhibitors, again you have very modest variation. It doesn't really make that much difference whose PBMCs you're using for the most part. However, if you use entry inhibitors, you commonly again see at least two orders of magnitude variation. So the amount of drug needed to inhibitor any given virus will be highly dependent upon whose PBMCs you are using. So this tells you that there must be postcell factors that impact entry inhibitor sensitivity again apparently. These are early studies, but it seems to a much greater extent than they do for other

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antiretrovirals. And this triggers a whole series of what I think are very practical questions that get at the use of these drugs and that I think will also tell us a lot about viral tropism and pathogenesis. So the questions are: What are the viral and host factors that influence entry inhibitor sensitivity, because if you could identify this, this information could potentially be used to help guide therapy. How might HIV acquire resistance to this new class of drugs, because it assuredly will. But importantly, what are the consequences of drug resistance? Do you get class resistance? If the virus becomes resistant to T20, does it become more or less resistant to other types of entry inhibitors such as coreceptor antagonists? And finally, how can we employ entry inhibitors most effectively? Because hopefully, within several years, we'll have a number of entry inhibitors from which to choose, and it would undoubtedly be foolish to use but a single entry inhibitor by itself, because you'll simply (unintelligible) for drug resistant viruses. So, what are some of the factors that can impact entry inhibitor sensitivity? Well, looking at T20, it is clear that the native envelope protein is resistant to T20. And on the far right-hand side, the six-helix bundle that snaps together conformation is also resistant to T20. But the virus becomes sensitive to T20 during a kinetic window that's opened by CD4 engagement and that is closed by coreceptor binding. So we hypothesized that

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viruses get fused more quickly than other viruses would probably be more resistant to compounds like T20, because T20 would have a shorter period of time in which to work. So what are some factors that can impact the rate of membrane fusion? Well, there are several that you can contemplate, and these each will probably be true in some subset of cases, so perhaps some viruses have more envelope protein on their surface than others. That would impact the rate of membrane fusion. Perhaps some are more easily triggered, requiring but a single receptor binding event rather than two or three. That would increase the rate of fusion. Perhaps some viruses undergo the conformational changes with higher fidelity than other viruses. We always depict these conformational changes as working all the time, but that may not be the case. There may be some viruses that are more fusogenic. When they engage receptor, they undergo these conformational changes more quickly and with higher fidelity. And finally, how about affinity for the receptors, because we know that there is wide variation in the affinity that different HIV strains have for their receptors. And we reasoned that viruses that bind to their receptors with higher affinity would fuse more quickly, because they would bind to their receptors more quickly as a result of enhanced affinity. So we tested this. And since we know a lot about how the envelope protein engages coreceptors, we pretty much knew what to do. So we examined the conserved coreceptor

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binding site, that I told you about, that plays an important role in modulating the affinity between the virus on the one hand and the coreceptor on the other, be it CCR5 or CXCR4. So what we did was to introduce single amino acid mutations in either a virus that uses CCR5 or CXCR4. And what we ended up with are a panel of viruses that are identical, except for single amino acid changes, that modulate the affinity of binding. Some of these mutations increased affinity, and some—most decreased affinity. And the specific mutations are not important. I just show three different views of gp120. And shown in light blue in the upper left and light gray in the bottom left and in green on the bottom right, there are some of the amino acids that we've mutated. And then we measure the consequences of this for virus infection. So all these viruses work, they're all infectious, but they bind to the coreceptor with different affinity. If you take these viruses and ask, well, how much T20 does it take to kill the virus, you find that the single amino acid changes in gp120, which is well removed from the T20 (unintelligible) in gp41, can impact T20 sensitivity by a hundredfold, by two orders of magnitude. So the wild type virus is on the right-hand side of this graph. Then most of the mutants that bind to receptors lower affinity require less T20 to inhibit the virus. So here's an example of a single amino acid changes in gp120 can significantly impact the potency of a drug that targets gp41. We see exactly the

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same results when we take these viruses and ask how much of the chemokine receptor antagonist do we need to inhibit membrane fusion. So what's the mechanism? Well, it turns out the mechanism has to do with the kinetics of membrane fusion, which we can measure using a fluorescent assay developed by Mike Miller at Merck. And so in this assay, you take the cells that you want to infect and you load them with a fluorescent dye that fluoresces green, so you've got a bunch of green cells. You then take the virus and incorporate into it an enzyme called beta-lactamase. When the virus gets into the cell, within several minutes the lactamase cleaves the fluorescent dye, so now it glows blue instead. So you can do this type of assay in a 96-well plate in a fluorimeter and watch the rate and extent to which your cells go from green to blue, and that's a good measure of the rate of virus entry. And if you do that with the mutants that I showed you, you see that the wild type virus, which required more drug to be inhibited, fused more quickly. That's the line to the furthest left, the dark blue line. And the various array of mutants that we have fuse now at different rates. They typically fuse more slowly. So when you reduce receptor affinity, you reduce the fusion, you give T20 a longer period of time in which to work. Another factor that impacts entry inhibitor sensitivity is chemokine receptor expression. So most of you here have between 50,000 and 70,000 copies of CD4 on the surface of your T cells, but

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they're not a CCR5 (unintelligible) be expressing your T cells will vary by at last a log, regardless of whether you have the delta-32 polymorphism or not. So to look at the effects of CCR5 expression levels, we took a cell line that had an inducible promoter, and so we have cells that expressed 10,000 or 50,000 or 90,000 copies of CCR5, shown in the different colored bars here. And then we infect these cells with HIV and ask how much drug do we need to inhibit. And what we find is that if you have more CCR5, shown in the red, you need more T20 and more coreceptor antagonist to inhibit the virus. So here the virus is the same. The cells are the same. The only variable is the amount of CCR5 on those cells, and that impacts drug sensitivity. And again--I want to show the data--this affects fusion kinetics. So if you have high levels of receptor, the virus fuses more quickly, T20 has a shorter period of time in which to work. So to put it together, T20 works during this kinetic window that's opened by CD4 binding and closed by coreceptor engagement, and factors that shorten the period of time that that window is open increase the amount of T20 needed to inhibit the virus, probably because it has a shorter period of time in which to bind. Factors that reduce the rate of membrane fusion will make HIV more sensitive to certain types of entry inhibitors such as T20. There are some practical implications associated with this that have to do with combination chemotherapy, and I think that understanding

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the mechanism of virus entry provides a very good theoretical basis for designing new clinical trials, and those coreceptor antagonists are used in combination with fusion inhibitors with this T20, because what the coreceptor antagonists do, they basically reduce chemokine receptor expression levels on the surface of cells. Now, CD4 binding will occur at the same rate, because you're not affecting CD4 levels by using the coreceptor antagonist. When you reduce coreceptor levels, it takes envelope protein trimers then a longer period of time to bind a sufficient number of coreceptors to cause membrane fusion, so, as a result, the T20 binding site is exposed for a longer period of time. So you would anticipate, and it turns out to be true in vitro, that the use of coreceptor antagonists and T20 result in synergistic inhibitions of HIV. And I think the mechanism has to do with the rates of membrane fusion. You slow fusion down, T20 works better. So we anticipate that there'll be a good reason to use T20 and coreceptor antagonists in combinations in clinical trials when the coreceptor antagonists reach the point in development where that can be contemplated. It also suggests that patients with lower levels of CCR5, for whatever reason, may respond better to entry inhibitors such as T20 and coreceptor antagonists than individuals who express high levels of chemokine receptors. This is at least testable, and it's something, I think, that should be looked at. So to put it together, HIV entry arises

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when sequential interactions between the envelope protein and T cell surface receptors that induce a series of conformational changes, each of which result in at least a transient exposure of highly conserved regions and envelope proteins that are otherwise sequestered and that play important roles in membrane fusion. Bristol-Myers Squibb has a small molecule--a paper describing it that's to be published soon--that binds to gp120 and blocks CD4 binding. There are a number of coreceptor antagonists that inhibit interactions between envelope and either CCR5 and CXCR4 that are in clinical development. And there are several fusion inhibitors that then block the final step—the formation of six-helix bundles. So, I think, from asking a very basic question, how does HIV go from outside a cell to inside a cell, this has provided important information not just about viral tropism and viral pathogenesis, but it's helped lead to the identification of entry inhibitors. And I think it's our job now to use this basic information to learn how to use these entry inhibitors in the most effective way possible. So I'd like to acknowledge in particular Jackie Reeves, a postdoc in my lab, who, with our team of technicians, basically does all of our work on entry inhibitors; our colleagues in the industry, Trimaris and Tikata (misspelled?) in particular, for providing us with their entry inhibitors that we've been able to use in our basic studies. And our work has been made possible by funding by NIH, by Ubaris

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(misspelled?) Welcome Fund, and by the Elizabeth Glaser Pediatric AIDS Foundation. Thanks very much.

JEAN-FRANCOIS DELFRAISSY: Thank you, Bob. Just an announcement. Please don't forget to sign the Declaration of Scientists and Physicians (unintelligible). This morning we obtained 2,000 (unintelligible) signatures. You must give your signed declaration to the staff with a red (unintelligible) before 3:00 p.m. Thanks a lot. Doug.

DOUGLAS RICHMAN: Thank you. Good morning. Our next speaker is Peter Rice. Peter is Associate Professor of Medicine at the Academic Medical Center at the University of Amsterdam. He serves as Deputy Director of the Dutch National AIDS Therapy Evaluation Center. Peter is Board certified in Internal Medicine with a subspecialty in Infectious Disease. He served on several European and U.S. committees, reviewing national clinical trials programs, including the ANRS and the ACTG. He's a member of a number of expert committees that are advising on opportunistic infections and complications of therapy. In addition to being a scholar, Peter is a true gentleman. Peter, it's time for your talk. And his talk will be on mechanisms and management of metabolic complications associated with highly active antiretroviral therapy.

PETER REIS: And let me also start by thanking the organizers and the scientific committee for giving me the opportunity to present to you. Today, I'm to talk on metabolic

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complications. We've known for quite some time now that, in fact, both exposure to nucleoside analogs and protease inhibitors contribute the onset of aberrant fat distribution as we see it in many of our patients. And the evidence for this comes from a number of studies, including both observational studies and clinical trials. One observational study performed by Simon Mallela (misspelled?) and colleagues in Western Australia and the Western Australian cohort showed that the risk of lipodystrophy was markedly increased in patients exposed simultaneously to nucleosides and protease inhibitors, in the red line, as opposed to patients exposed just to nucleosides, in the yellow dotted line. Similarly, in one of our own studies performed in the Netherlands and Belgium, the Promeseus (misspelled?) Study, we showed that patients simultaneously exposed to both classes of drugs, in the orange line again, had a higher risk of developing lipodystrophy than those exposed, in this case, to just protease inhibitors, in the blue line. If we then turn to the mechanisms that have been suggested by which protease inhibitors may be detrimental to adipocytes, adipocytes schematically shown here, the following evidence has appeared. First of all, protease inhibitors may interfere with the various transcription factors such as SREBP1, which are involved in the normal differentiation of adipocytes, thereby inhibiting differentiation. Second of all, protease inhibitors have been

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shown to interfere with the normal sensitivity of cells to the action of insulin, which, in this case, will lead to an increased activity of the enzyme hormone-sensitive lipase, which is crucial in regulating the breakdown of stored fat, resulting, in this case, in increased activity and increased breakdown of fat called lipolysis. Third of all, insulin also promotes the uptake of glucose into the cell by activating a receptor called glut-4, and protease inhibitors inhibit this process, thereby inhibiting the uptake of glucose, which may reduce the availability of glycerol for synthesis of triglycerides, and thereby decrease the possibility of lipogenesis or fat buildup. It is, however, questionable whether this mechanism will be very important in vivo. So, together, all of this will result in a reduced amount of fat volume. In addition to the mechanisms I've just alluded to, protease inhibitors have also been shown to stimulate the production of certain proinflammatory cytokines such as TNF-alpha and IL6, probably not only by adipocytes themselves, but also mononuclear cells interspersed within the adipose tissue, and these give rise to apoptosis (misspelled?), which again contributes to the loss of fat. It's important to remember, though, that when we discuss protease inhibitors, we sometimes tend to look at them as all being equal and belonging to the same class, but I think it's very important to notice that there may be marked differences in the mechanisms I just

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described between the various drugs, and this is something that I won't go into, but I'm sure will be discussed in the next session, both by Jacqueline Capeau (misspelled?) and Maureen Chamberlain (misspelled?) in vitro and in vivo. So I've shown you that there is resistance to the effect of insulin at the level of adipose tissue. But in addition to that, we and others have shown that there is also insulin resistance at the level of the liver and at the level of skeletal muscle. And, in fact, in the liver, despite the presence of insulin, glucose production remains elevated, there is increased hepatocellular (unintelligible). And similarly, at the level of the muscle, there is decreased uptake of glucose, decreased glucose metabolism intracellularly, and again, build up of fat. And both of these processes contribute to the onset of high blood sugar levels or hyperglycemia, which in some patients may lead to overt diabetes. So I've shown you that the resistance of the level of the fat cell leads to inability to store fat. In addition, there is an uncontrolled release of free fatty acids, and we know that free fatty acids entering the circulation will promote insulin resistance in these two organ systems. In addition to this, fat cells may produce less of certain hormones, such as adiponectin, and adiponectin itself has an insulin sensitizing activity, so reduced levels would again promote the insulin resistance in other organ systems. This has been demonstrated recently in a number of studies,

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including this study, which is from Finland by Sutine (misspelled?)and et al., and has been published, which will show that comparing patients with heart (misspelled?) and lipodystrophy to those without lipodystrophy, those with lipodystrophy had lower serum levels of adiponectin, and also in biopsies--in fat biopsies, had lower expression of adiponectin, Messenger, RNA. They were also able to show a correlation between both serum adiponectin and adiponectin, Messenger, RNA expression, and the amount of liver fat, suggesting insulin resistance at the level of the liver. In addition to this, a group from Denmark, at a recent meeting in the States, showed that they also showed similar findings but also showed that there was a correlation between the reduced expression of adiponectin and insulin resistance at the level of skeletal muscle, as demonstrated by the Clamp (misspelled?) Study. In addition to these effects, we also have learned that protease inhibitors may directly promote the onset of dyslipidemia, and the main mechanism suggested, although there is some uncertainty, is that protease inhibitors may promote the production and secretion of the LDL triglyceride by the liver. In addition to this, the effect shown on the left, which I've talked about with you just in the previous slide, where there's insulin resistance at the level of fat cells, will promote the free fatty acids entering the circulation, and it is known that free fatty acids in the circulation reaching

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the liver will also promote this process. Once you have higher levels of the LDL triglycerides, when these particles in plasma collide with HDL lipoproteins, the next change of triglycerides from here to here appears and of cholesterol esters from HDL to VLDL. And a similar process appears here. And all of this is mediated by the enzyme CuTp. What then happens is that the HDL triglyceride and the LDL triglyceride may be hydrolyzed by the activity of these two enzymes; in the case of HDL, leading to HDL particles shedding their major protein, apolipoprotein A1, which is then degraded by kidney tubular cells; and in the case of LDL, it leads to the formation of several small, dense LDL particles. And these processes, in fact, lead to a lipoprotein profile, which all through the various mechanisms outlined here, have a proatherogenic property and thereby contributes to the risk of increased atherosclerosis. In addition to this, we know that insulin resistance and also (unintelligible) distribution, both lipoatrophy and abnormal gain of fat will also contribute to this increased risk. What is much less clear and needs further investigation, I think, is to what extent HIV infection itself and the possible consequences of causing chronic vascular wall inflammation may contribute to this risk, and how this risk may again be modulated by providing anti-HIV therapy. An interesting study presented recently by Dressman (misspelled?) and colleagues in a neurine model, so this is in animals, showed that, in fact, protease

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inhibitors—several protease inhibitors, listed on the bottom of this slide, may contribute to atherogenesis in (unintelligible) of their effects on plasma lipids. What they showed is that when giving—providing these animals with lower doses of each of these drugs, shown in the left-hand bars, they could still show the promotion of atherogenesis in the aorta in these animals, despite the fact that plasma lipids were normal. And they have provided, I think, fairly convincing evidence that at least in these animals, this is promoted by an upregulation of the cell called CD-36 receptor on macrophages, and this receptor is involved in the uptake of oxidized LDL, and oxidized LDL uptake into these cells is really one of the first steps towards atherogenesis. However, it's questionable whether any of these effects may also be active in our patients, and this is something which needs further investigation. If we now turn our attention to the (unintelligible) which nucleoside analogs may be detrimental for adipocytes, the main mechanism which has been suggested is that nucleoside analogs may promote mitochondrial DNA depletion in these adipocytes, thereby leading to mitochondrial dysfunction, leading to a reduced scavaging of reactive oxygen species, which will dominate the mitochondria more (unintelligible), it will lead to leakage of certain components, and these components will induce the apoptotic pathway. In addition, at a later stage, there may be reduced ATP production, and as ATP in adipocytes is mainly used

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for triglyceride viral synthesis, this is reduced and lipogenesis is reduced as well. And again, this will contribute to the onset of lipoatrophy or may contribute to the onset of lipoatrophy. Again, I'd like to reiterate that just as for protease inhibitors, not all nucleoside analogs are equal in this respect. And this has been demonstrated very elegantly in an observational study, again in the Western Australia cohort by Mallela and colleagues, who showed that in naïve patients, commencing treatment with either D14 or a zidovudine-containing regimen, and looking at patients over time, examining their lymph fat by dexoscanning, they could show that there was a gradual loss of lymph fat over time, which, in fact, was a very early onset and then a gradual progression over time. And as you can see on this slide, this was more severe with D14 than with zidovudine-containing regimens. And similarly, in the metabolic substudy of the ACTG 384 trial, Dubet (misspelled?) showed that patients exposed to the DDI (misspelled?) D14-containing regimen, in the yellow curve, were more likely to lose their lymph fat than patients exposed to a zidovudine 3TC-containing therapy. It is, however, important to notice that although the effect was less in the zidovudine-containing arms, it was still present, and a gradual, although slower, loss of fat appeared. Studies such as these and other studies contributed to the potential of looking at interventions where certain nucleosides, such as D14

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and AZT, were replaced in a random fashion by nucleosides which, theoretically and from in vitro data, were less mitochondrial toxic, such as abacavir. And this is one of these studies, the Mitox (misspelled?) study, performed by Andrew Carr and colleagues in Sydney, where they initially reported in a randomized phase, where patients were either randomized to switch to abacavir or to remain (unintelligible) D14 and AZT, and it's important to note that most of the patients in the study were on the D14 rather than an AZT-containing regimen. In the first 6 months, and the results were published in JAMA last year, they showed that there was a significant, although not very marked, increase in peripheral lymph fat in those who switched to abacavir versus those who continued their treatment. However, last week, at the lipodystrophy workshop held here in Paris, Andrew Carr presented data after 2 years follow-up, and as you can see—this wasn't my doing. [music playing] We'll wait for a second. I think it was a nice interlude now. What they showed is that after 2 years, and this is very important and interesting, is that the regaining lymph fat in fact continued, and it may now be reaching levels which may be clinically important, although it's important to find out if the regaining of fat, if you look at it in absolute terms, is still less than the amount of fat patients probably lost in the first place. What happened to the other arm? To the other arm, patients were given the

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choice of either switching to abacavir as well or continuing their treatment, and the curve shows the combined arm. And what you should know, what is not shown in the slide, is that the patients who opted to continue the D14 (unintelligible) really had a flat curve; whereas, there was a regaining of fat starting in those switched to abacavir. Another intriguing possibility is that NRTI-induced mitochondrial toxicity may also possibly contribute to insulin resistance at the level of skeletal muscle. And this is circumstantial evidence which comes from a study performed in people without HIV--in fact, in healthy people, and recently published in Science, where a group of elderly patients, or elderly people, I should say, in their early 70s, were compared to a group of young people in their late 20s, who were matched for body composition. And what these investigators did, is they assessed insulin resistance by performing euglycemic hyperinsulinemic clamps in those groups, and they also assessed mitochondrial function in muscle by performing (unintelligible)trostopy. What they found is that they, during the clamp studies, that the rate of peripheral glucose uptake indeed was reduced in the elderly compared to the young, suggesting insulin resistance. As expected, this was associated with an increased intramyocellular lipid content in the elderly, in their muscle. And they could also demonstrate that mitochondrial oxidative as well as (unintelligible) relation capacity were reduced. They

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also looked at lipolysis, and they, in fact, demonstrated that whole body lipolysis as well as regional lipolysis were not different between the groups. And therefore, their argument is that increased release of free fatty acids is not contributing to this process, and they suggest that it's, in fact, mitochondrial dysfunction which is directly responsible for the skeletal muscle incident resistance. They also go on to argue that, in fact, with age there may be increasing mitochondrial DNA mutations, and this may lead to mitochondrial dysfunction, and that may be the pathway to insulin resistance. Mitochondrial DNA copy number, however, in skeletal muscle cells, does not change with age, as shown in this study recently published from Australia. However, with NRTIs we may speculate that mitochondrial DNA depletion may occur in muscle cells and, in addition, NRTIs, as has been shown by the group of Mallela (misspelled?), and they also, over time, with treatment, induce mitochondrial DNA mutations. So both pathways possibly, although I would highlight this is speculative, may contribute to this process. It has become clear that the potential for effective intervention is limited, because, as I outlined, various direct adverse effects of the drugs that we use, of lipid and glucose metabolism, are reinforced once (unintelligible) distribution is established. And this may explain the relative lack of success of interventions, which are aimed at reversing metabolic

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abnormalities and glucose intolerance in particular. This was shown in one of our studies, the results of which were presented by my colleague Mark (unintelligible) yesterday, or it was last week, at the lipodystrophy workshop also. And we studied a group of patients who were well suppressed with their HIV infection on a protease-containing regimen, in whom we replaced the protease inhibitor by nucleoside analog abacavir. And we studied—these patients were studied extensively by euglycemic hyperinsulinemic clamps, both before the switch and at several time points after the switch, out to 2 years after switching. If you look at the slide, then you can see that in normal volunteers, endogenous glucose production, as well as lipolysis, will be suppressed to a marked extent under hyperinsulinemic conditions during the clamp. However, in patients on a protease inhibitor-containing regimen, first of all, their fasting endogenous glucose production, if you compare it to volunteers, as well as lipolysis is more elevated, and what you can see is that, during the clamp, there is much less suppression than in the volunteers. And, unfortunately, shown here and here, 2 years after protease inhibitor withdrawal, there is some, but certainly not any improvement in the direction of what you find in volunteers, suggesting that there is persistence of insulin resistance both in adipose tissue and muscle or in the liver. The same is true, in fact, for peripheral glucose uptake, shown from the

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same study in this slide. What happens normally, in normal volunteers, is that under hyperinsulinemic conditions, glucose intake will go up markedly. And as you can see, this does not happen in the patients with lipodystrophy, both while they're on PI as well as when they've replaced their PI for 2 years. Fortunately, when switching patients, the effect on plasma lipids may be more beneficial, and this is shown in this study, which is the metabolic substudy from the Spanish (unintelligible) study, which, I believe, is soon to be published in the New England Journal. And what these investigators did is they took patients on a protease inhibitor-containing regimen and were well suppressed, and they randomly allocated to switching their protease inhibitor for either abacavir, shown (unintelligible) in black, or nevirapine in red. And as you can see, somewhat dependent on the type of switch, there was a reduction in both total and LDL cholesterol as well as triglycerides, which was less so with abacavir switch, and it also showed the intriguing propensity of nonnucleoside-containing regimens to increase HDL, and somewhat more so with nevirapine than nefavirin (misspelled?), something which we already have shown previously in patients first commencing treatment. So I think it's clear that a more successful management of the arrangements in particularly glucose but also lipid metabolisms in patients with established (unintelligible) distribution will depend on our ability to

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reverse the actual redistribution of fat. Several interventions have been tried, and most of these, such as recombinant human growth hormone or metformin, overall have yielded fairly disappointing results, although both of these interventions may be useful in patients with isolated fat accumulation, but unfortunately those patients are very rare. In addition, both interventions, although they may reduce central fat, unfortunately, at the same time, they may also reduce peripheral fat. In addition, growth hormone may promote insulin resistance, although with lower doses, this is less of a problem, and it may transient, but it's still not really what you want to see happening. A newer strategy, which is being tried and has been tried now in several clinical trials, is the intervention with the clypisone (misspelled?) class of drugs, and these are insulin sensitizing agents, which also have the intriguing propensity to promote fat cell differentiation. So theoretically, it's a very interesting class of drugs to investigate. And there are now two controlled studies, randomized controlled studies, which have been represented, and they're consistent with a study from Finland, which has been published, and the study presented, I think, yesterday, and also at the lipodystrophy workshop by the Grinfund (misspelled?) in Boston, and presented by Colleen Hadigan (misspelled?). Both studies showed that insulin resistance was, in fact, markedly improved by these drugs, by

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(unintelligible) liposome, in this case; but whereas, the (unintelligible) study from Finland did not show any improvement in fat distribution, the study from Boston did. And I think what this tells us is that we need to study these interventions further and particularly need to identify which patients stand to benefit the most from this intervention. So, all in all, treating fat free distribution, once it's there, is not very promising and very difficult. So, can we and should we not focus more on trying to prevent these metabolic complications as well as their potential atherogenic consequences in patients who start treatment for the first time. There are several options that we can think of, such as using nucleoside-sparing (misspelled?) regimens, employing so-called least toxic PIs, or PI-sparing regimens employing least mitochondrial toxic nucleoside or nucleoside analogs, using combinations of least toxic PIs, least toxic nucleosides. Also, particularly in patients at high risk of cardiovascular disease, we may exploit the potentially less atherogenic lipid profile of NNRTIs. And, of course, only the future will tell us whether novel drug classes, for instance, such as have been discussed in the previous talk, will have a lesser propensity to cause fat distribution. And fortunately, there are several trial as outlined by Patrick Yeni, in his plenary talk yesterday, which are investigating this. But I think it's very important that all of these trials should make sure to

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investigate lipodystrophy and fat distribution by objective measures, so that at the end of the day, in these trials, we will really know which of these strategies and regimens are more beneficial to our patients. In order to improve the prevention, particularly the prevention and also the treatment of these complications, we urgently need to further improve our understanding of what actually drives this fat distribution, and look at mechanisms by which these drug classes and also the individual drugs exert their effects. Genetic susceptibility probably comes into play, etc. So in the last part of my talk, I'd like to throw another idea at you, which, I think, is one of the unexplained issues. And one of the unexplained issues in my mind is that it's not really clear why fat disappears in certain regions of the body and it increases in other parts of the body. And several explanations have been suggested, such as that there may be a difference in regional expression of certain receptors in the endocrine environment and the mitochondrial content or mitochondrial susceptibility to these drugs, or in drug penetration in various adipose tissue compartments and handling of these drugs by these compartments. But another possibility, central, another possibility that we are thinking about is that the brain may actually play a role. We've known for quite a while that, in fact, substances produced by fat cells have an effect on the brain in regions particularly of the hypothalamus and (unintelligible) nucleus,

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where they affect a number of receptors, and through activation of downstream pathways, they will, in fact, influence both energy intake and eating behavior, as well as energy expenditure, as well as fat, and also, as has recently been shown, glucose metabolism. What has been much less clear is how signals from the brain go back to fat. This has been studied in animals and, in fact, by a group in Amsterdam at the Institute for Brain Research. And what they showed is that not only is there—there is, in fact, innervation of fat cells from the autonomic nervous system, coming from brain stem and hypothalamus, and there's not only, as has been shown in the past, sympathetic nervous innervation of fat, which is known to have catabolic effects in leading to fat loss, but what they also were able to show is that there is parasympathetic innervation on fat. These studies were done by taking a rat model and injecting transneuronal tracers, such as pseudorabies virus, into specific fat depots, and then following the infection as it proceeded upwards towards the brain through the urinal system, and then make it visually—show it visually by using various fluorescent dyes. Not only were they able to show that neuroanatomically fat is innervated by both the sympathetic and parasympathetic nervous system, but it also has functional consequences for the behavior of fat. And I'm showing you one example of those studies, which is what they did is they actually surgically selectively denervated a fat

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pad—in this case, an intraabdominal fat pad on one side of the animal, but not on the other side of the animal, and then performed euglycemic hyperinsulinemic clamps on these animals, and looked at what happened. And, in fact, after parasympathetic denervation, they show a marked decrease in glucose uptake and free fatty acid uptake into these fat cells, which would then prevent these fat cells from storing fat, and at the same time, they showed an increase in the activity of hormone sensitive lipase, which, as I've told you before, is responsible for breaking down fat. This was after parasympathetic denervation. So what this suggests is that, in normal circumstances, parasympathetic output from the central nervous system will promote fat storage and inhibit fat (unintelligible), which is, in fact, the opposite of what had already been shown by others for sympathetic activity. Importantly, they also were able to show that looking at these regions in the hypothalamus and brain stem, that there were so-called somatotopy (misspelled?) of this sympathetic and parasympathetic innervation. What this means is that within the sympathetic and the parasympathetic nuclei was an (unintelligible) there of discrete sets of neurons which project to the intraabdominal fat and other sets of neurons which project to the subcutaneous fat. And this offers the possibility that the central nervous system, in fact, can selectively control adipose tissue in different parts of the

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body, and it can do so by balancing out (unintelligible) from the sympathetic and the parasympathetic nervous system. But in addition, and this is very important, it must also be able to selectively control this output with respect to specific fat compartment. And, of course, one of our not so good national newspapers got part of the message, but not the complete message, and wrote up this paper in an editorial with this cartoon, which I thought was very funny, and therefore decided to show it to you. So what we've done recently, and this will hopefully soon be published in Lancet, is coining the hypothesis that the actual aberrant distribution of body fat that we see in our patients with HIV and lipodystrophy may, in fact--and this is a question mark--represent selective autonomic neuropathy. And the idea is that antiretroviral therapy--and I want to notice that some of the components in the drug that we use, such as DDI and D14, are, in fact, neurotoxic agents and have also been shown to carry a high risk of lipodystrophy—that antiretroviral therapy may, in fact, selectively influence these sets of neurons in the brain, thereby creating an imbalance between the sympathetic and the parasympathetic output and possibly leading to the loss of subcutaneous fat and the gaining of visceral fat. And it's interesting to note, in this respect, that the particular regions of the brain where these neurons lie is, in fact, very accessible. There is no blood brain barrier present in that

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particular part of the brain, which makes it accessible not only to natural substances, which is important because, as I've told you, that, in fact, natural substances produced by fat cells need to enter the brain, but it may also make it more accessible to certain of the drugs that we use. So, on this note, I'd like to end, but not before thanking a lot of people. And as you can see, I have also, during this talk, markedly changed my fat distribution. And I'd like to thank not only colleagues of mine in Amsterdam and elsewhere in the Netherlands, particularly Mark (unintelligible) and Eric (unintelligible), but also the many collaborators in other parts of the world that I have the pleasure to collaborate with, such as Simon Mallela and David Nolan in Perse (misspelled?), Andrew Carr and David (unintelligible) in Sydney, (unintelligible) Linford and (unintelligible) Miller in Copenhagen, Jacqueline Capeau here in Paris, Lucy Sutine in Finland, and, of course, the always continuing support of (unintelligible). Thank you very much for your attention.

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