DR. KATHY PRITCHARD: Thank you very much, Soon. And it’s my pleasure to introduce Dr. Mitch Dowsett. Welcome, Mitch.

DR. MITCH DOWSETT: Thanks very much, Kathy. It’s a real pleasure to be here at this really interesting to me. [Slides are put up] The task I got to do today is really to review the measures, proliferation and apoptosis, as being measures of response in neoadjuvant treatment.

I’m going to… [tries to advance the slides] So, I’m going to cover two main topics here today -- chemotherapy and endocrine therapy -- and talk about, particularly, changes in proliferation and apoptosis as measures of response. I’ll deal with the other types of novel therapies just in one slide at the very end.

In going through this, I’ve chosen not to try to do a comprehensive review of all of the literature, but really to base most of the comments I’ll make on our own data. I’m focusing on relatively small but detailed sets of observations, because I think that’s probably more meaningful in this situation.

This is the sort of tissue that we use and I think we all get. We’ve used these 14-gauge core cuts for many years now. They weigh -- if you get a good core cut, which this, of course, is -- about 30 (13?) milligrams. And you can get up to 30 micrograms of RNA from one of those. The average we get when we use RNAlater and take a section from it for DNA and for an H&E is more like 10 micrograms.

I’m going to be talking in terms of proliferation mainly about Ki67 -- or “Kee67” as we call [pronounce] it in the UK -- and apoptosis measured by the TUNEL method. TUNEL, of course, actually works by linking in residues onto the short ends of DNA which are created by the apoptotic process. And you can see -- this is a classical cell -- it’s actually in the presence of a field of normal, untreated cells.
Key point in this is that only about one percent of cells are apoptotic in an untreated breast cancer. We score about 3,000 cells for that, but that only gives us still 30 as our main number. So, there’s an error in this. Just for completeness, I thought I would mention that, of course, there are other methods for measuring proliferation than Ki67. People have used S-phase, mitotic index, of course, thymidine labeling index, the bromodeoxyuridine. We did actually review this in a *JCO* article a couple of years ago. And all of those really link into proliferation in a relatively similar fashion and correlate in a relatively good manner.

In terms of apoptosis, probably the gold standard, in fact, remains morphology. And, certainly, when we apply the TUNEL method, we always actually apply morphology on top of that.

And whilst Ki67 may be actually susceptible to image analysis in a relatively easy fashion, I’m not sure that apoptosis is. Other ways of measuring apoptosis include activated caspase 3. This M30 -- which is actually a serum assay. There are other methods -- or other components of cell death -- we also need to consider. Autophagy, which is actually just now beginning to become well characterized. Possibly necrosis, which might even be localized necrosis. I was talking to Sandy Swain just briefly about the data that came from the bevacizumab -- which I always pronounce wrongly -- data yesterday. Well, there seems to be local -- sort of clusters of -- apoptosis; or you might even call it local necrosis around the areas of the vessels.

These are some of the data we derived early on, which just show you the error that you get from measuring Ki67 or apoptosis by TUNEL; but, two core-cut biopsies from the same lesion at the same time, so no intervening treatment. And you can see here that the mean error is around about 33 percent. The mean… the standard deviation error is 16
percent. That’s actually an approximation because the error envelope here is in fact of this sort of shape. And really, that just gives an approximation.

But it’s useful, though, for you to think about, because essentially, that means that for any two measurements to be different from one another with 95 percent confidence, you need a 50 percent difference. And for apoptosis, it’s nearer 60 percent difference.

And I’m going to show you a lot of data with lines joining up measurements from individual patients. But you need to think, I think, around about that… that measure envelope around each of those points.

Another thing to just think about is the relationship there is between apoptosis and proliferation in untreated tumor. This is 220 patients from the IMPACT trial, which I’ll talk about later. You can see there is a very strong, highly significant, really strong relationship between Ki67 and apoptosis. And we found a rho value which varies between 0.5 and about 0.65 in just about every subset of tumors we’ve looked at.

So, in fact, if you look at apoptosis as a univariate, it actually -- high apoptosis is an indicator of poorer outcome, which is actually counter-intuitive.

So just thinking about chemotherapy and some of the observations we’ve made there -- we talked yesterday about high proliferation pre-treatment being an indicator of good response to chemotherapy -- so, a good predictive factor, but, actually, looking at poor long-term outcome in relation to this, an essentially a poor prognostic factor.

I think most of us really would agree, if you can get a pathological complete remission in a patient with a high proliferation, what we’re really doing there is perhaps eradicating, in at least some of those patients, disease which would have otherwise been indicating poor outcome overall in that group of patients. So, one really needs to think about this
continuum when you’re trying to think about response or not in the neoadjuvant chemotherapy situation.

Data in terms of apoptosis in chemotherapy… and most of the chemotherapy I’m talking about here… I’m just talking about as chemotherapy. This is AC or other anthracycline-based regimens in the main. 24 hours after starting chemotherapy, you can see there is a substantial increase in the apoptotic index from this mean level of about one percent – highly statistically significant. And you also get a bit of a rundown in Ki67. It is, in fact, significant here. You can see it’s variable. It’s fairly modest -- about 15 percent, overall. Neither of these, in our hands, have linked well into predicting response in the individual patient.

Looking at proliferation over a longer period and looking at the excision here, you can see clearly that, by the time you get to excision, which is -- this series was between three months and 18 weeks after starting treatment -- virtually all patients do show some degree of reduction in proliferation, but you still get some that don’t change at all.

I’m not going to show you data on apoptosis in this circumstance. We have data on that, but, really, the data out at excision is, I think, confusion. It’s contradictory. And, in part, that’s because many of these patients have, as you’ve seen in the pathological presentations, very few cells to work with. So, getting 30 thousand -- sorry, 3,000 -- cells from that to get just 30 cells positive is really pretty tough, and we’ve had variable data.

In terms of the relationship with clinical response, I’ve tried to just summarize it here. So, thinking about proliferation and the pre-treatment and 24-hour change, as I said -- no real significant relationship there. But Andreas Makris is in the audience here, Jenny Chang and Laura Assersohn, who are not with us, did individual series. And all of these showed significant relationships between the 21-day reduction in proliferation and clinical response. In terms of apoptosis, Jenny did a preliminary study with us, which
was reported as significant, using an unusual methodology involving flow cytometry. When we used a rather more rugged methodology, with Marina here -- Marina Parton -- we really weren’t able to confirm that.

Now, these data are the first time I’ve shown these data in public, and I think these will make up a small part of an ASCO presentation later this year. And, it now looks at the actual Ki67 in the excision specimen here and the matched pretreatment from those same patients. One thing, of course, to think about here is that none of these patients can be clinical… sorry, complete pathological remitters or, indeed, near-complete pathological remitters, because we’ve got excision biopsy to work with.

So the Ki67, as you’d expect, does have a relationship. These are tertiles -- you can see the cut-offs here based on the levels in the pretreatment specimen -- a highly significant relationship. But when you get to the excision specimen, we really have radically poorer outcome in this high proliferative group. So, again, I guess this is emphasizing that if you’re high proliferation and you do not show a pathological complete remission, you’re really looking pretty bad indeed. And, again, these might be patients, as Soon was talking about, that would be candidates for a post-neoadjuvant randomized study of some sort.

Moving through to endocrine therapy -- and I’d say we’ve actually focused rather more on endocrine therapy in recent years. And speaking to you about the IMPACT study, which is a study which we’ve learned a great deal from. This, as Ian was saying yesterday, is the randomization of anastrozole versus tamoxifen versus a combination. And, really, we tried to mimic as best we could the ATAC protocol, to see whether we could be predictive of the ATAC outcome here.

We have biopsies pre-treatment, at two weeks, and then out here at 12 weeks. And you can see the numbers here. The biopsy at two weeks was not mandatory, but we got 159
samples from that, all the same. The ATAC outcome, as most of you will know, is that we see this improvement of anastrozole over tamoxifen; and the combination being very similar -- I think a good control, in this circumstance. We really wanted to know whether we could predict that.

Firstly, commenting on apoptosis, though, in this series: We found, against expectations really, that apoptosis was not increased in these patients, and we looked at this very thoroughly indeed. Indeed, it was decreased with all three of the arms at two weeks, and at 12 weeks for anastrozole as well.

And in fact, Ian referred to study 223 yesterday, which is an anastrozole-alone arm… - alone study… at least in one arm. And we were able to confirm that there. Apoptosis actually decreases to some extent with the aromatase inhibitors. And I think that’s largely linked into that slide that I showed you earlier on, where you have this strong relationship with apoptosis and proliferation.

Clearly, the decrease in proliferation, which is shown here, markedly outweighs any decrease in apoptosis. And if you care to construct this so-called cell turnover index, which is a ratio of the two -- will show you get markedly decreased values.

So, these are the [Ki67] data just on the anastrozole. I could show you very similar data for tamoxifen and the combination. Profound changes by two weeks, largely the same at 12 weeks. And we focused a little bit on this yesterday -- as to whether or not these patients are recovering. We really don’t know whether they’re recoverers, or whether they are within that error envelope I was talking about. Overall, the correlation between the 12-week values and the two-week values is about 0.6.

The overall data, which were really quite encouraging to us, clearly -- Ian, again, showed yesterday -- show you that, at two weeks, anastrozole suppresses [Ki67] by 76 percent;
out here, at 12 weeks, by 80 percent. And in each circumstance, not only was it better than tamoxifen, it was also better than the combination. And therefore we felt did indeed predict the outcome of ATAC.

What we didn’t do was actually predict clinical response in this same setting. Now just focus here at the all patients -- putting everybody together here at two weeks and at 12 weeks. The blue bar shows you the responders and a somewhat greater suppression of Ki67 than in the purple bar [non-responders] there, but not statistically significant in this projected analysis. Now I can squeeze, I can massage those data, and I can create a statistically significant result for you. But, clearly the result is actually… there is no close relationship.

In this audience, this is a very, very naive slide to show. But I think it’s something which has helped me think about how to think about these markers and what their relevance is to clinical response.

So this tries to illustrate a tumor which is growing rapidly in the absence of treatment. In the presence of treatment, this rapidly growing tumor is impeded in its growth, but it does not regress. It is a non-responder -- it’s progressive disease by common terms. But proliferation, of course, is markedly suppressed in that circumstance.

Here’s another patient. This patient, actually -- slower-growing, similar in overall (no?) response. But in this case, because she was slower-growing, she creates a response. She’s now a partial responder. And the Ki67, of course, is decreased.

If we link this to recurrence-free survival, we would expect the prognosis of both these to be somewhat poorer than here. But in both cases, the actual benefit from treatment comes through, because we’re jacking up the line -- these patients are expecting to actually recur less quickly.
So, essentially, a change in proliferation is likely, I think -- when I say, “I think”, I think it is rational to expect -- a change in proliferation to predict outcome in the adjuvant setting somewhat better than predicting response in the neoadjuvant setting.

Just briefly looking now -- the two-week data in more detail. There are 56 patients on here, 52 of whom show some reduction in proliferation. So, there seems to be a continuum in benefit here. You can see that the changes are very variable. Some of the patients at the highest levels in particular show very modest reductions in proliferation, but others show profound changes in proliferation. And this got us sort of thinking over the last 12 months or so. It had to perhaps conceptualize the Ki67 at these various time points.

And the way we sort of started thinking was that, well, perhaps baseline Ki67 will be expected to be prognostic, as we would all expect. And the change between the pre-treatment and the two-week would be predictive of the benefit of treatment from that particular agent. And then the two-week value, then, might integrate this sort of intrinsic prognostic value with the predictive value, such that this point out at two weeks -- it wouldn’t matter where you started from, but it would matter where you ended up, as long as you’re on that particular endocrine treatment. So, in rationally, the two-week Ki67 should relate to outcome on anastrozole more closely than baseline Ki67.

And these, of course, are the data -- we published these in *JNCI* just, I think, about a month or so now -- a month or so ago. Tertiles, again, of, now, lnKi67, showing you that the… at baseline, the patients with the highest Ki67 have the poorest outcome, as expected. But then when we look at the two-week lnKi67, the separation is much cleaner, much greater; the p-value is smaller.
But, most importantly… I mean, I’ve shown you those as tertiles -- the analysis was based as a continuous analysis. When we put all of this in together and say, the pre-treatment value’s in, and the two-week value’s in -- what actually stays in the multivariate prediction of recurrence-free survival? Well, tumor size is in there, and two-week Ki67, but NOT pre-treatment Ki67. And, interestingly, two-week ER, but not pre-treatment ER remained in there.

Now, this is the thing we’re going to be looking at further in this POETIC trial, which Ian described -- really trying to see whether we can confirm whether these two-week measurements are more predictive -- or more accurate -- in prediction than at baseline.

Now just lastly, I wanted to show you a couple of slides from a study in which we are measuring proliferation, but some other features as well. This is one of these window-of-opportunity studies which I know Matt [Ellis]’s going to talk about a little bit more shortly. And what we’ve done here… there’s 35 estrogen-receptor-positive patients in here. They received anastrozole or letrozole -- it doesn’t matter very much as far as this analysis is concerned. And then we ended up using a cDNA microarray, with 17,500 features, looking at both the biopsy and the excision sample.

One of the interesting things here is, when we throw all those 70 samples into the microarray -- and those 17,000 features each -- and do a clustering analysis, we pull apart about half of the pairs so that… there’s 18 of the pairs stay together, here, at the first level of clustering, but then 17 are pulled apart.

And, over here, in this arm -- you won’t really be able to see it here -- but the “B”’s are the two-week sample. 20 of the 25 lines there are “B” samples. So, this treatment seems to be making the samples much more like post-treatment samples than like their partner, pre-treatment samples, as far as these are concerned. That’s something we’ve seen much more [of] with endocrine therapy than we’ve seen with chemotherapy.
Just to illustrate I think the power of this study, we’ve constructed the most naïve bioinformatics tool you can get, which is called -- we’re calling it the Global Index of Dependence on Estrogen, or “GIDE”.

And that is just counting the number of genes which change up or down -- not worried about the direction -- beyond a given threshold. And we’ve just chosen two-fold here. And I’m going to show you how that index relates to the concentration of estrogen receptor and HER2 status.

The GIDE, as shown here… obviously I’m not looking for you to actually see the details here, but, essentially, we’ve just ordered this by the highest GIDE down to the lowest GIDE.

And I think it’s just worthwhile reflecting for a second: All of these are estrogen-receptor-positive patients. All of them have received an aromatase inhibitor. Yet, in this case, we’ve got 4,000 genes change. And, in this case, 79 genes change, within the breast tumor. And a continuum throughout.

In terms of the estrogen receptor -- the green values here are the nine with the highest estrogen receptor levels, the red, with the lowest. Separating, I guess as we’d really expect, with some of the high estrogen receptor levels not getting a particularly good response; none of the patients with a low estrogen receptor level’s getting a very good response. And the four amplified HER2 cases here are down with low GIDEs.

Now, that’s a very naive treatment of the data. I think it’s interesting to just think about in terms of its showing this continuum. But, currently, we want to break into this now, and try to understand the features that make up that GIDE. Proliferation is going to be one of them. (It’s going to be about one minute, Kathy, I think.)
Proliferation is one of them. We would expect, I think, other features to be important in determining the outcome of the patients, and that’s what we want to try to get that here with the POETIC trial in particular, by getting large numbers of patients with pairs of samples where we can look at the molecular makeup of those.

We can determine the estrogen dependence of the feature. We can hopefully determine the importance of the clinical benefit of changing that feature by estrogen deprivation. And, hopefully, in the pre-treatment samples, we can look at the determinants of response of that feature to estrogen deprivation.

This POETIC study, if it comes off, will actually involve 4,000 patients, 2,600 of which will have short-term pre-surgical aromatase inhibitor treatment.

So, just trying to summarize -- as far as chemotherapy is concerned, decreased proliferation is associated with response to treatment. High residual Ki67 is associated with a very poor recurrence-free survival. And increased apoptosis occurs in response to therapy, but there doesn’t seem to be a close relationship with response.

In the endocrine treatment situation, decreased proliferation may predict for treatment benefit, but, again, there’s no close relationship with clinical response. High two-week Ki67 is associated, again, with poor recurrence-free survival. An increased apoptosis does not occur at two weeks or 12 weeks with tamoxifen or aromatase inhibitor.

And, lastly, just this word about novel agents -- I’ve just talked about endocrine therapy and chemotherapy here. I think, though, with the endocrine agents we’ve seen, there have been very consistent effects. I think we need to be open-minded about which of these features which determine response are going to be affected by the growth factor inhibitors, the anti-angiogenic agents, etc. Early… early studies, detailed studies, will
determine how we actually utilize these as markers of response. Okay, thanks for your attention.