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Dr. Schwartz, Dr. Stone, dear colleagues, good evening. I first would like to thank the organizers, in particular Dr. Naghavi, for the invitation to participate in this symposium tonight and to be a part of the faculty. My task is to review some of the more recent biomarkers that may represent valuable additional diagnostic tools in the primary assessment of risk in initially healthy subjects and, since inflammation plays such an important role in atherosclerosis, I would like to restrict my talk to markers of inflammation. Probably most of you in here know about C-reactive protein. So, I will briefly give you an update on some more recent findings and I also will briefly show some data of other markers that are emerging and then focus on one more recent marker that I think is a very promising one.

You have seen probably a similar slide by other speakers, but I would like to show it again and set the stage for my talk just by saying that office-based risk assessment probably still is what we are doing today and what we should do in the future. And by whatever score we use, be it the Framingham score or the PROCAM score, we will divide subjects into those at high risk, intermediate risk, and low risk. You can see the proportions of the subjects that fall into these categories. About 25 percent are at high risk, and about 40 percent are at intermediate risk. So, really, a relatively large part of the population falls into the intermediate risk category and about 35 percent into those at low risk. Based on Framingham, for example, this is below 6 or 10 percent, between 6 and 20 percent, and above 20 percent. Now, we have clear guidelines on

what to do with those at high risk and those at low risk. Certainly, we'll reinvite the latter and we'll follow them. But what to do with those at intermediate risk? Our basic strategy is shown here and we have heard a lot about other tools that may be of value as additional tests. You see here, the pre-test probability and risk of the CHD over 10 years and here's the post test probability. If you pick out, for example, someone with a 10-year risk of 15 percent, he would be placed at an intermediate risk. If you submit such a person to an additional test, be it an imaging test or a measurement of a biomarker and you come up with a positive result, then you may be able to place him in a high risk category; or if this test is negative, you would take him down to someone at low risk and wouldn't do anything further.

This is a more or less complete list of biomarkers related to inflammation that have been studied prospectively in large epidemological studies over the years and you're probably familiar with the vast majority of those. There are some old markers and several more recent ones. You may also realize that this list is somewhat different or does not incorporate, for example, some of the markers that have been shown by Dr. Serruys. I strongly believe this makes sense because in the primary care setting we are interested in markers that predict long-term risk and these markers clearly may be different from the ones you use in patients with chest pain in the acute coronary syndrome. But just to give you one example. I think there is encouraging data that soluble CD40 ligand may play a role in the acute coronary syndrome, but there is no data so far that supports its use in initially healthy subjects.

Here you see some non-protein markers and there are data around for almost 30 years showing that increased leukocyte count is associated with cardiovascular disease outcome. Then you have the more frequently studied proteins here and probably the largest databases are present for C-reactive protein and obviously fibrinogen; however, fibrinogen didn't make it into a clinical scenario because standardization is incomplete and as long as this is the case it remains at this stage, although, data are around for about 20 years now. The first study was published in 1984 from Gothenburg. Then, for example, PAI-1 has also been tested prospectively, but it may be more related to the metabolic syndrome and may play a more important role in diabetics. Then there are some data on markers of endothelial function like, for example, von Willebrand Factor and we have already heard about cellular adhesion molecules. There were some very interesting initial, data but a more recent meta-analysis has shown that the association between these markers and outcome is not really encouraging. And there is a whole variety of cytokines;. we have already heard about those. I will pick out one which I think

is of interest, namely IL-18. And then on the right side, you see some more exotic parameters that are not used routinely, but by and large, all of these markers you'll see on that slide, as I've already said, have been tested prospectively. They have been measured once in the vast majority of these studies and have been consistently related to various cardiovascular disease outcomes. And finally, you see here Lp-PLA_2 on which I'll elaborate a little bit later on.

Coming to C-reactive protein, you're aware of this data. Almost 20 studies, long-term studies, have been published so far. They're listed here and you'll see the relative risk on the right side together with the confidence intervals; by and large if you're in the top quartile of the distribution of C-reactive protein, you have a twofold increased risk as compared to the bottom quartile.

Now, this data has drawn considerable attention during the 2002 American Heart Association meeting. It came from the Women's Health Study published by Paul Ridkar's group, and in that study, the authors compared the predictive value of C-reactive protein to the main lipid marker, LDL cholesterol and you see here various levels of adjustment -- the crude and age adjusted and risk factor adjusted analysis, and here the marker is given in quintiles. What you see if you compare these figures up here, 2.3 and 1.5, is that C-reactive protein may be superior to the LDL in predicting future risk. Although, if you look at the area under the curve, there is not really a difference; however, additional statistical testing has revealed that there might be such a difference.

This slide you have already seen. Basically, what we wanted to show is if there is any additional value of a marker over and above what we already can yield from the various scores. This attempt has been first undertaken by Paul Ridker in the same paper, in the *New England Journal*. You see here the Framingham estimate of 10-year risk and the multivariable relative risk, and this is further divided into C-reactive protein based on the AHA/CDC recommendation. Here you see that in those at intermediate risk, there seems to be some variation if you stratify the Framingham risk score by C-reactive protein.

Based on the abundant evidence of an association between CRP and cardiovascular disease outcomes, at the beginning of last year, there was an AHA/CDC statement based on a Consensus Meeting that suggested that among the current inflammatory markers identified, CRP measured by a high sensitivity assay has analyte and assay characteristics most conducive to use in practice and was a Class 2A level of

evidence B and at the same time and the same meeting, other markers of inflammation were reviewed and the panel came to the conclusion that these other markers should not be measured for determination of cardiovascular risks in addition to high sensitivity CLP. This however, may change over time, when we get more data on other markers.

After this initial data from Paul Ridker's group, also in 2003,there was a negative study that aimed at looking into the value of C-reactive protein in addition to the Framingham score and that data came from Rotterdam. It was a nested case-control study within a population based cohort of about 8,000 men and women over 55. There was a relatively weak association which was not statistically significant between CRP and outcome if the Framingham score was assessed, with and without C-reactive protein. And you do see here the results, the basic risk and then the risk functions. With CRP there are two different models and you see there is no difference between the model containing CRP and the one without CRP. Now, one has to say that this population was predominantly elderly subjects, which is one point; the second point is, if you look at the data, C-reactive protein in cases was unusually low compared to all other published data. And the third point is that in one of these models, the authors have included left ventricular hypertrophy and this obviously, indicates subclinical disease. So, I'm not really surprised that the CRP didn't make it in that study.

We had the chance to look into the same issue in the MONICA Augsburg Cohort in 3,435 men, aged 45 to 74 years, who were followed over six and a half years and those were all men randomly selected from the general population. They were participants of three different MONICA surveys, and similar to the slide that showed Paul Ridker's data, you do see here the Framingham estimate of 10-year risk in percent. This indicates the number of events in the various Framingham categories. Here is the population at risk and again, you see those at intermediate risk between 6 and 20%. This is a relatively large part of the population under study. What you would expect with various Framingham categories, you'll see an increase in the multi-variable relative risks. Now, on the right side, we did the same thing as Paul Ridker has already done. In this male population, we divided the various Framingham risk categories by C-reactive protein, again using the recommended cut points 1, 2, and 3, which have been published in *Circulation* and what you see here first and this is indicated by the so-called AIC (Akaike's Information Criteria) -- is a better fit of the model if you include CRP. This is what these figures mean, if there's a difference of greater than 10.

What you see on the right side here is that obviously there is no additional value of C-reactive protein in those of very low risk, but if you look here at those at

intermediate risk and particularly between 10 and 20%, there is a considerable modification of the multi-variable risk, if you stratify for C-reactive protein. This is particularly true for those at 15 to 19 percent, and there is a trend in those at high risk, but this was not statistically significant. To make it a little bit more difficult, we tried to do some additional statistics. We divided the Framingham score into three categories, and into five categories. Here are the events and the population at risk, and here you see the relative risks and the confidence intervals. This is the model without CRP, and this is the model including C-reactive protein. Again, you see a better fit indicated by differences in Akaike's Information Criterion. You see a significant change-in-estimate in excess of 10%, which is also an indication that the model including C-reactive protein performs better. And finally, you do see a significant increase in the area under the curve in ROC-analysis. All three different statistical approaches do point in the same direction and obviously indicate that C-reactive protein gives additional information over and above what we know from the Framingham risk score.

Here are the conclusions of our study. I didn't show the first data, elevated CRP concentrations and elevated total cholesterol over HDL cholesterol ratio. They're both independently related to incident CHD, similar to what Paul Ridker in the Women's Health Study has found; and addition of CRP to a prediction model of total cholesterol over HDL cholesterol ratio, and the Framingham risk score results in a better fit of the model containing CRP and significantly improves prediction of incident CHD, and this was particularly true for those at intermediate risk, namely between 10% and 20% over 10 years. Thus CRP may modulate coronary risk and may, therefore, modify the physician's interpretation of the patient's risk; however, this applies to many of the data we have seen tonight. Clearly, these findings must be replicated in other studies.

The second marker, I briefly want to touch upon is Interleukin 18. There was some initial data in subjects with manifest atherosclerosis showing that elevated interleukin 18 levels are associated with total mortality and with recurrent coronary events. But only recently there was some data from the PRIME study which comprised two populations from France and from Belfast in initially healthy subjects, a relatively large population and the design was again, a case-control study. Here are the relative risks and here are the IL-18 in tertiles and you see an increase in risk with increasing tertiles for the combined end point which included angina and here is only coronary death and MI. Now, the interesting thing with IL-18 is that we know it plays a crucial role in the inflammatory cascade and it has been found in the atherosclerotic plaque, and in the

experimental setting inhibition of IL-18 was associated with a decrease in atherosclerotic lesion size. I'm showing you that IL-18 was largely independent of other markers, that is CRP, fibrinogen, IL-6, and so on.

Finally, I'm coming to the phospholipases and I would like to touch upon Lp-PLA_2, the Lipoprotein-associated Phospholipase A_2. It's a platelet-activating factor acetylhydrolase. Dr. Serruys has already mentioned it in his talk. It's a 50 KDa, Ca-insensitive lipase produced predominantly by the characteristic cells in atherosclerotic plaque, namely macrophages/monocytes, T-cells, and mast cells. The important point is that this enzyme generates pro-inflammatory substances and namely, lyso-PC or oxidized free fatty acid from oxidized LDL and these inflammatory products may be important in the atherosclerotic process. There is experimental data showing that an inhibitor of Lp-PLA 2 is associated with an anti-atherosclerotic properties that decreases lesion size. This has been demonstrated in a rabbit model and obviously, the interesting question is whether or not plasma levels correlate with CHD in patients. Now, this slide shows you the same thing. The enzyme generates these products, lyso-PC, oxidized free fatty-acid and they're involved in basic processes that are important in atherosclerosis, namely in the attraction of certain molecules, monocytes, and also in migratory processes.

The first clinical evidence, in favor of an association between Lp-PLA_2 and coronary heart disease came from WOSCOPS. You're obviously familiar with that study in primary prevention, hyperlipidemic men, about 6 1/2 thousand, with no previous MI followed-up of 5 years and the main result showed the efficacy of pravastatin in this primary care setting in reducing coronary events. The design of that study was a nested control design, 580 coronary events and 1100 event-free controls from this population. And as I said, they were followed for about five years and were randomly selected and matched for age and smoking. Now, the basic result showed that Lp-PLA_2 and other markers of inflammation like fibrinogen, white blood cell count, and C-reactive protein in univariate analysis, predicted coronary risk. This was the risk associated with a one standard deviation increase of the respective marker. If you adjusted these analyses for further inflammatory markers, then first fibrinogen dropped out. The others were still significant. If you further adjusted for all conventional risk factors, then in this study only Lp-PLA 2 stayed as a significant predictor for future coronary events.

Just a couple of weeks ago, results from ARIC were published. In that population Lp-PLA_2 was also assessed using a case-cohort design with 680 individuals with

incident CHD who were compared to 740 controls from a cohort random sample. Lp-PLA_2 was measured by the same test as in WOSCOPS, supplied by diaDexus, the PLAC test, and high sensitivity C-reactive protein, in that case by the Denka Seiken assay. The population is characterized in this slide. There was a relatively large proportion of diabetics among cases, further the population was moderately obese and showed relatively low total cholesterol levels. I'd like to remind you in the WOSCOPS' study, the mean total cholesterol was about 275. So, relatively low total cholesterol, moderately or elevated average LDL, normal blood pressure in ARIC, and you see a difference in Lp-PLA_2, as there is also a difference in C-reactive protein between cases and controls. In overall crude analysis given here in this model, there was a significant association between elevated levels of Lp-PLA_2 and coronary heart disease outcomes; but in the ARIC study, after adjustment was carried out for age, sex, and race and conventional risk factors, this was no longer significant; however, if you looked at those with an LDL below 130, there was a significant association with about a two-fold increase in risk. And finally, if you looked into the fully adjusted model, additionally adjusting for C-reactive protein, the association was about the same size with a twofold increase in risk. So, in that study, there was a particular positive association in those with relatively low LDL. The lack of association between the Lp-PLA 2 and various conventional risk factors is of special interest. Since the Lp-PLA 2 travels with LDL in blood, it's not surprising that the only association is with LDL. This is a positive association and a negative association was seen with HDL and obviously except, fo total cholesterol by and large, there is no relevant further association with other conventional risk factors. This again shows the association of Lp-PLA_2 and C-reactive protein with coronary risk in patients with low LDL cholesterol and I think this is really a stratum of the population that deserves our particular attention. You'll see here, it's about a three-fold increase risk if the Lp-PLA_2 is elevated and the C-reactive protein is elevated too. So, clearly Lp-PLA_2 seems to be of additive value.

Finally, we also had the chance to look into that association in a large cohort. Again, it's a population from Southern Germany, the MONICA Augsburg Cohort. More than 900 middle-aged men, 45 to 64 who participated in the first MONICA survey in 1984. Since we wanted to study incidence events, we excluded prevalent CHD. Cardiovascular risk factors were collected in a standardized manner. Lp-PLA_2 was measured by the same test as in ARIC and in WOSCOPS and the end-point determination was based on MONICA criteria. We used only hard end points, namely fatal and non-fatal MI and sudden coronary deaths, so angina was not an end-point.

This is our population, a relatively obese population. Total cholesterol is between WOSCOPS and ARIC. The HDL is similar to ARIC. Blood pressure's slightly higher and I think there are somewhat more smokers than in ARIC. And again, you see a clear difference between cases and controls with regard to Lp-PLA_2 and also with regard to C-reactive protein. We performed similar analysis, looked into the correlation between of Lp-PLA 2, CRP, and other risk factors and again, you see for Lp-PLA_2, by and large a lack of correlation with other risk factors, except for the total cholesterol in our population. Whereas, for C-reactive protein you see a correlation with systolic blood pressure, and in particular with body mass and smoking, which is not shown here.

Since the ARIC data have not been published at the time when we analyzed our data, we carried out analyses similar to those published by the WOSCOPS` group. First we looked into a model, which contains only C-reactive protein and a model containing only Lp-PLA_2. We did different adjustments, first no adjustments, then adjusted for diabetes and smoking and finally multivariable adjustments were done. What you see here are relative risks associated with a one standard deviation of the marker and you see it's all significant. The same applies to Lp-PLA_2. Again, I would like to stress that in that analysis, only one of the markers was in the model at one time. Now, obviously, the second thing we had to do was putting both markers into the same model and we proceeded in the same way and adjusted our analyses for age, diabetes and smoking and further adjusted for other risk factors that are listed here at the bottom; and you see if you look at the multivariable model that, despite the fact that both markers, CRP and Lp-PLA_2 are in the model together with conventional risk factors, both independently predict the future risk of coronary heart disease.

And finally, we looked into potential additional value of Lp-PLA_2 to C-reactive protein and formed four groups. One with low CRP, that is below three milligrams per liter and low Lp-PLA_2, this was the tertile cut-point; then obviously either C-reactive protein or Lp-PLA_2 was elevated or as seen here in gray, both were elevated. And again, three levels of adjustment are presented; and what you'll see is a consistent finding that those with high Lp-PLA_2 and high C-reactive protein showed the highest risk which was significant in all three models. So, clearly, in this analysis, an additive value of Lp-PLA_2 could be shown.

Summary

Finally, I would like to summarize and I hope I didn't take too much of the time. Lp-PLA_2 was the strongest predictor and biomarker of coronary events and was

independent of traditional and emerging risk factors including CRP in hyperlipidemic individuals. That was the main result of WOSCOPS. In the ARIC study in particular, in individuals with low LDL that means below 130 milligrams pro liter, levels of Lp-PLA_2 were independently associated with incident CHD in multivariable analysis, including CRP. Our MONICA cohort showed that Lp-PLA_2 was predictive of coronary events in a population-based sample of initially healthy middle-aged men with moderately elevated total cholesterol during long-term follow-up of 14 years. The two earlier studies had a mean follow-up of about 4 to 5 years. So, in addition to C-reactive protein, which I think is fairly well established, Lp-PLA_2 appears to be a further promising marker of atherosclerotic complications and deserves further study.

Thank you very much for your attention. (Applause)