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This is the podcast from *Clinical Chemistry*. I am Bob Barrett. Studies of disease associations with γ' fibrinogen, a newly emerging risk factor for cardiovascular disease, have been hampered by the lack of a standardized, well-characterized assay.

The May issue of *Clinical Chemistry* reported on an immunometric technique to measure γ' fibrinogen concentrations in plasma and the clinical utility of this test in samples from healthy individuals enrolled in the Framingham Offspring Study and in a controlled study of coronary artery disease.

The article reported that γ' fibrinogen shows promise as a marker for cardiovascular disease. The addition of this marker to other established risk factors, such as high sensitivity C-reactive protein and cholesterol, may provide another predictive value for assessment of risk of adverse cardiac events.

Dr. Steve Kazmierczak, Director of Clinical Chemistry, and Dr. David Farrell, Professor, Department of Pathology at the Oregon Health & Science University, are coauthors of this report. They are also our guests in this podcast.

Tell us, Dr. Farrell, why did you study γ' fibrinogen?

Dr. David Farrell:

We have been studying γ' fibrinogen at the biochemical in my lab, because I am a biochemist, and we have been interested in its structural properties. And we found that this forms blood clots that are resistant to breakdown essentially.

So that got us to thinking, well, if you had too much of this in your blood, it actually might predispose you to some sort of thrombotic disease. So in some ways we went about this backwards from the way an epidemiologist would normally approach a disease.

In an epidemiologist's approach, you have got a disease and you look for the risk factors that contribute to that disease. Well, in a sense we had what we thought would be a risk factor for some sort of thrombotic disease, and we looked for which diseases it might cause.

So the ones that we have been looking at so far have been coronary artery disease and heart attack and stroke, and deep vein thrombosis. So the results that were in the *Clinical Chemistry* paper from Case Control Study of coronary artery disease that we completed, suggested that there was in fact a very

strong association between the levels of γ' fibrinogen and coronary artery disease in this Case Control Study.

So it somewhat validated our original thinking that because of the properties of this clotting factor, the fact that it would make these blood clots that were resistant to breakdown, that if it was too high, it may in fact predispose you to thrombosis.

Host: Well, now, what exactly is γ' fibrinogen, and how is it similar or different from total fibrinogen?

Dr. David Farrell: Right. So γ' fibrinogen is a subtype of total fibrinogen. Now, fibrinogen is the main structural protein in your blood that forms the basis of the blood clot. If you clot pure fibrinogen, it will set up to look something like jello.

So γ' fibrinogen is a structural protein that is a form of total fibrinogen, but it has an altered chain in it. So fibrinogen has three types of polypeptide chains that make it up: the A α , the B β , and the γ chains, and there are two copies of each chain. So fibrinogen is assembled as a disulfide-bonded dimer.

Now, in the γ' form, what you have is an alternative mRNA processing event in the γ chain mRNA. And so about 10% of the time, and that amount varies from person-to-person, so somewhere between, let's say, 5-10% on average, the γ chain, instead of going through its normal slicing at the mRNA level, there will be a polyadenylation site within the last intron, intron 9, that's recognized by the polyadenylation enzymes.

So what they do then is polyadenylate within what would be the non-coding intron, and it cleaves off the three prime end to the messenger RNA. When it does that, you lose the splice site that you would normally get to cleanly excise intron 9.

So what happens when this is translated by the ribosome is it reads into what was a non-coding intronic region and there are codons for 20 amino acids in that section.

So in the γ' chain of fibrinogen, the carboxyl-terminus has 20 amino acids that are different from the usual four amino acids that are in the more common γ chain, which is often referred to as the γ A chain.

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So what we have studied is how much this varies from person-to-person, and it apparently changes ratios quite a bit. So the ratio of γ A chains to γ' chains varies quite a bit from person-to-person. And it's the functional properties of that γ' chain that we think are important.

So in the carboxyl end of the γ A chain you have binding sites that are normally involved in platelet aggregation. Well, in the γ' chain, those are missing. But what you have in its place is a high affinity thrombin-binding site. And we think that that may have something to do with its altered clot properties, it is having this extra gain of function, if you will, at the C-terminus.

I should stress that this is not just a polymorphism or a mutation that occurs, everybody has a certain amount of this γ' fibrinogen splice variant to a greater or lesser extent. So it's not like a polymorphism in a population, as you would have with say Factor V Leiden. It's going to be in everybody to a greater or a lesser degree.

Host: Okay. Well, with that in mind, how much γ' fibrinogen does a typical person have?

Dr. David Farrell: Typically, γ' fibrinogen makes up about 10% of the total fibrinogen on a mass basis. Keeping in mind that in γ' fibrinogen, what you usually have is one γ' chain paired with a γ A chain. So since fibrinogen is a dimer, you have two A α chains, two B β chains, two γ chains.

And because the γ' chain is made at about a-tenth or less of the level of the γ A chain, and the chains are assembled randomly into fibrinogen molecules, that means that most often the γ' chain is going to be paired with a γ A chain. So if you have about 5% of the chains being made as γ' , then that's going to mean that 10% of the fibrinogen molecules are going to be this γ A, γ' , heterodimer.

And there is probably a small amount of γ' chain homodimer fibrinogen, but it's going to be at maybe a-tenth of the γ' , γ A level, just because of the random assortment, the way these two chains come together.

But what we found from the Framingham Offspring Cohort is that the actual concentration varies

tremendously from person-to-person, and that's something that really we didn't expect, because if you look at the concentration of most clotting factors, they are really kept within a fairly tight range of maybe two or maybe three-fold at most.

So most of the other clotting factors, if you have major changes in the concentration of the clotting factor, you get either frank thrombosis or you get hemophilia type of disease.

So we were very surprised to find that γ' fibrinogen, which is itself a clotting factor, varies up to 40-fold between individuals, and that came out of examining the Framingham Offspring Cohort, which had about 3,400 or so different individuals. And we were quite impressed with the very wide range of variability.

And since we had already known that it has different biochemical properties than the major form of fibrinogen, we thought, well, this could in fact be an underappreciated potential risk factor for thrombosis, and it could vary quite a bit from individual-to-individual. And unless you actually had an assay to detect it, you would never know it.

Host: Well, how much does γ' fibrinogen vary within each individual?

Dr. David Farrell: Well, that's something that Dr. Kazmierczak can address, because we have been collaborating on an experiment to look at that.

Dr. Steve Kazmierczak: Thanks, Dave. Well, unfortunately, there is really no good data on the intra-individual variation of γ' fibrinogen. However, there actually is some data on the intra-individual variation in total fibrinogen in two recent studies that were done.

One, which measured total fibrinogen monthly for five months, in healthy individuals, found that there was approximately a 13% intra-individual variability in total fibrinogen.

And another study, which was a much shorter duration, this was measured over a two-month period, reported a 14% intra-individual variability in total fibrinogen.

You can compare this to total cholesterol, which most studies suggest that total cholesterol shows a 7% or less intra-individual variability, that is how much the

marker or analyte will change within a particular individual on a day-to-day basis.

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And this is really important to look at, especially for prognostic risk assessment factors, because the clinical utility of these factors is oftentimes related to how much variability. If the marker has too much variability, the clinical utility drops off dramatically.

And currently, as Dave mentioned, we are in the middle of a year-long study, where we have 15 individuals that are drawn weekly for a month, and then on a monthly basis up to a year. And what we are looking at with this study is trying to compare total fibrinogen, γ' fibrinogen, high-sensitivity, or high-sensitive CRP, as well as total cholesterol, HDL cholesterol. And our goal here is to compare all these different markers that are used for prognostic purposes and really try to stack them up and see which of these markers has the greatest variability and which has the tightest intra-individual variability. And that's something that we, actually this study should be completed by the end of August of this year.

Host: Are there any environmental or biological parameters that regulate γ' fibrinogen levels?

Dr. David Farrell: Yeah, there are both environmental and genetic components to γ' fibrinogen regulation. Like total fibrinogen, γ' fibrinogen is an acute phase reactant and increases with inflammation.

In fact, one of my former graduate students, Dr. Chantelle Rein, investigated the roles of different inflammatory cytokines on γ' fibrinogen expression in a human liver cell line to try and identify effects that are directly applied to the liver cells compared to other ones that, say, may be through intermediate to mediator, such as monocytes, for example.

But there are genetic polymorphisms that are associated with either increased or decreased levels of γ' fibrinogen that have been identified. So there is a fibrinogen 9340 T>C polymorphism that's associated with increased γ' fibrinogen levels, whereas there is another one, the 10034 C>T polymorphism that's associated with decreased fibrinogen levels.

So in fact, it's a mix of both environmental and biological parameters that regulate γ' fibrinogen levels. One of the things that we have currently undertaken with the Framingham Heart Study is to identify transacting SNPs that might be involved in the regulation of this, doing a genome-wide association study based on data from the Framingham Offspring Cohort. So we are hoping we can identify other genetic components that affect γ' fibrinogen regulation as well.

Host: As a marker for cardiovascular disease, is γ' fibrinogen simply a surrogate for total fibrinogen, and is there a correlation between total and γ' fibrinogen?

Dr. Steve Kazmierczak: Well, that's a very important issue, because total fibrinogen levels are already a known and established risk factor for heart attack and stroke. But in our analysis, the Framingham Offspring Cohort, γ' fibrinogen didn't show a strong association with two markers that are known to associate with total fibrinogen levels, which are systolic blood pressure and total cholesterol.

So if γ' fibrinogen was simply a surrogate for total fibrinogen, you would expect that it should correlate with systolic blood pressure and total cholesterol as well, but it does not.

And we have published a previous case control study of coronary artery disease, the data of which was used in the Receiver Operator Characteristic Curve Analysis in our *Clinical Chemistry* paper. We have previously published data from that study showing that the association between total fibrinogen levels and γ' fibrinogen levels in that study had a *P* value of only 0.2, suggesting that they are not surrogate markers.

And in fact, in a more recent unpublished study of periodontitis patients with underlying cardiovascular disease, we also saw no significant association between γ' fibrinogen and total fibrinogen.

So it appears that they are not simply surrogate markers and that they may in fact have additive predictive values, looking at each one of them together. So in fact, we may get more information by looking at the two of them together than with either one alone.

Host: How is γ' fibrinogen associated with other established cardiovascular disease risk factors?

Dr. David Farrell:

Well, in our examination of the Framingham Offspring Cohort we saw that γ' fibrinogen was significantly associated with several established CVD risk factors, particularly age. It was associated with female gender. It was associated with Body Mass Index, with cigarette smoking, diabetes, fasting blood glucose, and triglycerides. And it was inversely related to cardioprotective HDL cholesterol levels.

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What I found more interesting was the risk factors that it's not associated with, which were systolic blood pressure and particularly total cholesterol. This may mean that we can pick up people who have normal cholesterol levels and think that they have a clean bill of health, as far as cardiovascular disease goes, and yet still at risk of a heart attack, because as you know, many people with normal cholesterol levels still have heart attacks and yet when you go to the doctor's office and get a cholesterol test done, it comes out with a normal range. You may feel that this is a bill of clean health, and we know that's simply not the case. The total cholesterol levels are just part of a complete analysis of your risk assessment.

So we are hoping that this is going to be another marker that will add more predictive power to better identify people who are at risk of a heart attack and particularly those that might have normal cholesterol levels.

Host:

Okay. So what are the limitations of your assay and the data presented in the paper?

Dr. David Farrell:

I will turn that one over to Dr. Kazmierczak, who is our Head of *Clinical Chemistry*.

Dr. Steve Kazmierczak:

Unfortunately, as with all studies, there is limitations and ours was not immune from any of these limitations. What I see it is as four limitations.

Number one is with the Framingham Offspring samples, these were samples that were collected between 1998 and 2001, and then frozen for roughly the past ten or more years. And the reality is that the stability of γ' fibrinogen as well as any other marker that's measured in previously banked and frozen sera, the stability for a lot of markers is really not known. So that could be viewed really as one limitation of this study.

And this sort of comes back to a point that Dave had made earlier is that, we looked at the distribution of the γ' fibrinogen in the Framingham Offspring samples, we found that the distribution was significantly skewed when compared to samples obtained from blood donors. And the skewness of the Framingham samples could be due to the age of the samples and that may be there was some degradation in the γ' fibrinogen in some samples, or more likely it could be that many of the samples that we are obtaining from these Framingham patients may have had underlying sub-clinical cardiovascular disease that had not manifested itself as an acute event yet. So even though these patients were classified as having no cardiovascular event, they still did have underlying cardiovascular disease.

Another limitation is that both the samples from the Framingham Offspring Study as well as the Coronary Artery Disease Cohort, these samples are retrospective and really the real test of any prognostic marker is really to see how it performs when measured prospectively in the individual patient.

Epidemiologic studies are great for teasing out markers that may show differences between different patient groups, but the real test is to see how a marker is going to perform in that individual patient. That's something that we still need to look at.

The third limitation is that, when we look at both the Framingham Offspring samples as well as the Coronary Artery Disease Cohort, the patients from whom these samples were obtained were relatively older. The CAD cohort had a mean age of 62 years as well and the Framingham Offspring patients that are samples from the patients enrolled in that study were 61 years. So it would be nice to evaluate this test with a much broader distribution of age ranges and not just look at the older patient population.

The final limitation, and this is something that we are actively working on now is that, the method that we used for measuring γ' fibrinogen really didn't have the precision which we would really have liked to see.

For example, a normal range that we determined for γ' fibrinogen of 0.1 to 0.5 grams per liter, the imprecision of the method at a γ' fibrinogen concentration of 0.1 was approximately 20%, and was somewhat better at the upper end of 0.5, where

it was possibly 5% of our cutoff threshold, which showed that the greatest diagnostic accuracy of the test still had an imprecision of roughly 10%.

But even given all these four limitations, the area under the ROC curve for differentiating our cases versus controls showed an area of 0.76, with a diagnostic accuracy of 0.78.

So still with all these limitations, the test did perform actually very well for differentiating patients with and without coronary artery disease.

Host: Okay. So in your opinion or opinions, how do you see this analyte being used in five years?

Dr. David Farrell: Well, I would envision this analyte being used in addition to our current testing for traditional risk factors for heart attack risk assessment. For example, markers like cholesterol and triglycerides and fasting glucose levels.

And hopefully, this will allow us to achieve more accurate risk assessment and particularly be able to identify people who are at risk of a heart attack, but are missed by the current test. I focus particularly on cholesterol, because of the lack of association between γ' fibrinogen and cholesterol, and the idea that perhaps this could help us identify those people with normal cholesterol levels who are in fact still at risk of a heart attack and think that they have actually got a clean bill of health.

Host: Dr. Steve Kazmierczak is Director of *Clinical Chemistry*, and Dr. David Farrell is a Professor in the Department of Pathology at Oregon Health & Science University. They have been our guests in this podcast from *Clinical Chemistry*.

I am Bob Barrett. Thanks for listening.

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