FS: Good afternoon and welcome to the second in the series of Critical Path Rounds. I'm Carolyn Wilson from the Center for Biologics and I wanted to introduce the speaker for today, Dr. Abdu Alayash. He received his Ph.D from the University of Essex in the United Kingdom, worked in a variety of places, including King Faisal University in Saudi Arabia and Duke University, before joining CBER in 1989. Currently, he is the Chief of the Laboratory of Biochemistry and Vascular Biology and has regulatory oversight for the review of blood substitutes. In his 20 years' experience with FDA, he has received numerous awards and notable especially for this particular venue are the receipt of both CBER and FDA Scientific Achievement Awards.

As you will hear today, Dr. Alayash's laboratory is now working on the biochemistry of hemoglobin-based oxygen carriers or HBOCs. He is trying to define an animal model that will have improved correlation with the indigo impact of HBOC in human clinical studies. The availability of a more predictive preclinical model should enhance the ability of manufacturers to move the field forward in an iterative manner screening for toxicity prior to human exposure, saving both time and money and, in the end, leading to more safe and effective life-saving products. The work that Dr. Alayash will present today provides an excellent example of CBER's Critical Path Research Program where CBER scientists perform research to address key regulatory issues to facilitate product development.

DR. ABDU ALAYASH

Thank you, Carolyn. The presentation today will focus on some of our CBER Critical Path research, which I refer to here as discovery science in

relation to hemoglobin-based blood substitutes. These are unique and complex products that have been under development for the last 30 years. Unfortunately, we still do not really have an approved product yet. The program or my lab was established almost 20 years ago and the whole idea is to provide the reviewers, CMC, preclinical, and the clinical with some understanding of the product and, of course, also to contribute to the review process. Okay, hemoglobin-based blood substitutes, also referred to as HBOCs, are not really substitutes for blood; it's a misnomer. But they do basically provide oxygen delivery and volume replacement. And they do not obviously perform other functions of blood in terms of protection against infection or nutritional aspects and so on and so forth. All they provide is, of course, oxygen; quantity of oxygen, and volume replacement. The advantage, of course, you don't really have to worry about compatibility; they are universal in terms of blood matching, long-term storage and, of course, this would appeal to the army, or in case of, God forbid, disastrous situations, and it is pathogen free.

You could basically make them [blood substitutes] virus-free or pathogen free, available in case of emergency and, of course, like I said, the army and the navy have great interest in this product. We have basically two classes of products, the hemoglobin-based and the fluorocarbon-based product. I'm not going to talk about the fluorocarbons. These are synthetic compounds. The hemoglobin-based, which are the focus of my research and really the bulk of the regulatory review, these products are derived from outdated blood. Hemoglobin is isolated, purified and then hemoglobin either chemically cross-linked, cross-linked and/or the surface of the hemoglobin is

enlarged by adding a non-protein component. In some cases, the hemoglobin is polymerized.

In other cases, the hemoglobin is actually encapsulated in a form to make it like the red cell. The basis for this is really very simple--two purposes. The manufacturers tried to do the following. Chemical reagents normally tend to stabilize hemoglobin in the tetrameric form. As you know, if you take hemoglobin out of the red cells in dilute medium, it will break apart into dimers. And of course, if you tried to infuse that, it will be cleared by the kidney and it will be very toxic. So these chemical reagents, what they do [is] basically put it back together in the tetrameric form; they can enlarge the surface. It can be even made much longer to stay longer in circulation and, of course, like I said, it can be actually encapsulated to mimic the red cell.

This is a list of some of the sponsors or manufacturers and again just to remind you, this is actually based on what is available in the literature. So again, the manufacturers here, the product and as you can see, the manufacturers generally use the same approach, either a cross-linked and cross-linked or polymerized products. There are one or two actually recombinant hemoglobins. They started out in *E. coli* and expressed the protein. Clinical indications, as you can see, a variety of indications from surgery to trauma; a couple, of these sponsors unfortunately are no longer with us.

So what I'm going to do is basically try to choose at least three or four examples where our research targeted a number of issues or problems that

are of relevance to the review of these products, from CMC, pre-clinical and, of course, clinical aspects. I'm going to start first with the product characterization; this is very important because we're dealing with compounds, that we have no precedents. We don't really have anything within CBER or anywhere else that we can compare it to. These proteins have undergone extensive chemical modifications. In some cases twice, two chemical processes. So we needed to know what effect these chemical modifications will have on the protein in terms of stability, and in terms of safety.

I have chosen here an example, where how one sponsor predicted in terms of chemistry or the characteristics of the given product and what we have found out later—it was totally completely different chemistry. And then I'll go through the list one by one. The product for your information, the manufacturers gave us this product eight, nine years ago and we've done all the paperwork in terms of the legal aspects. Everything you see here has been published and presented publicly. I'm going to refer to the product as poly-HbA, which is presumably a polymerized hemoglobin. It is a large hemoglobin. We found out the chemistry that the manufacturer had actually put in the product didn't turn up what they anticipated or predicted.

And the chemical reagent was not specifically targeting the protein. It went all over the place and that led to destabilization and ultimately [to] the unsafe use of this particular product. And here is a summary of what happens. I mean, you're looking at the hemoglobin molecule. Like I said, orange is the the alpha subunit and the grey is the beta subunit. The manufacturer, in this case you used sugar, which is a trisaccharides. They

put the sugar through a process to open up the rings and they mixed the reagent with the hemoglobin in oxygen-free environment. And they predicted, or they did at the time, using molecular modeling and a number of HPLCs and have convinced themselves and of course they convinced us at the time that this reagent actually goes into this space, this is known as the 2,3 DPG pocket, and presumably here are the three crosslinking points, two on the beta subuints and one on the alpha subunit. So it has cross-linked the protein and the reagent in the same time cross-linked amino acids on the surface and polymerized the protein. So the product is cross-linked and polymerized. And if you actually look at the size exclusion chromatography that we did ourselves, it looks truly a polymer. But when you look at the oxygen equilibrium curves (OECs), now this from your classic biochemistry of hemoglobin, normally this is hemoglobin or red cells, and when you look at the oxygen equilibrium curves (OECs), of the blood, fresh blood, it's nice and sigmoid (S-shaped).

And when you take the hemoglobin out of the red cell and purify it, or take it out of the red cell, the curve will shift to the left, which means it will deliver less oxygen. As for the product in question, if you look at the curve, it doesn't even look anywhere near the red cells or the free hemoglobin. It's almost linear. It does not saturate where the oxygen is full and obviously we have raised this question earlier with the manufacturer. They will say, well, this is a new physiology; we don't have to mimic the red cells. We have animal data, human volunteer data, and so on and so forth. Then, like I said, they gave us the product, and we worked on it.

We did things that we know manufacturers would not really do with the technology available at the time. So the first abnormality we recognized was the heme itself. Now the heme, if you'll recall, is basically made up of four rings with the metal (iron), referred to as porphyrin. Using EPR, we found that, actually, the iron is tilted and that will put some constraint on the heme. So the heme could easily break up within the protein. The other abnormality we found is that the hemoglobin, even when you cross-link it, it still carries oxygen and delivers oxygen. So the hemoglobin should be able to go from one form to another, even when you chemically modify it.

In this case, what we found out is that actually the hemoglobin, in this particular case, is locked in one form. In other words, it would not unload oxygen quite regularly. Then we went on to do protein analysis. We've literally broken down the protein, using a variety of amino acid analyses and digestions of the protein. What we found out, there were the reagents in question… actually [it] didn't go where it was supposed to go. It was supposed to go here [in the DPG pocket]. Like I said, remember what they told us that it was supposed to be cross-linked and that at the time, actually, they did present some very fancy graphics, which of course the computer can tell you anything you want, in a way.

You don't have to sort of play around with the program. But that's not really the point. What we found out [is] that the reagent, this green fellow here, is actually not even here but on an amino acid, which is very close to the heme. And here is the heme. And here, we did the calculation to convince ourselves and everybody else that not but the reagent itself, in fact, part of it, part of the sugar went on this very point. Just to give you a close up,

again, remember the reagent was supposed to go here with three point cross-linkages. It didn't go. It went down here. It's very close to the heme and we believe this is what led to destablization, and this led to that abnormal geometric or rather abnormal geometry of the heme and so on and so forth.

The product was withdrawn from the market for further development. Not because of this, but the clinical picture didn't look very good and the product was dropped at the time. The manufacturer then came to us and we helped them in [the] design of hemoglobin but, unfortunately, of course, financially, they could not really push any further. So that's one story. The next issue is potency and this I'm sure you all struggled with, and how do we define potency and particularly with a product such as this, which carries oxygen. Straight forward, of course, you look [at the] oxygen curve that I showed you before. And couple that with some animal data and of course that would convince us and everybody else that the product will do what it's supposed to do.

We were able, as I will show you in a moment, to help identify a marker that helped us both and hopefully industry, a more precise than conventional methods. Now I'm sure you're all familiar when somebody comes to you with a product, particularly a hemoglobin product, and we normally ask for in vitro data and of course it would be nice if you coupled that with in vivo and if the two will match, in [the] case of hemoglobin and of course we ask for oxygen work such as the one I showed you, it would be nice if we can actually show that in [an] animal to show that it actually delivers oxygen and/or hypoxic condition or what have you.

Now remember again we're dealing with a product, unlike any other biologic that we've dealt with simply because of the heme and again remember the hemoglobin has the heme iron, which undergoes oxidation. You can't really control it. You can't stop it. It's very similar to rusting and this is exactly what happened biologically. And of course what happens, if you leave the hemoglobin out, it will oxidize rapidly. It will start oxidation. Now this will happen of course in the red cells. But remember, the red cells; we have a number of enzymes that reduce it back to the normal functional form. In the process of course you will also produce radicals that could damage the hemoglobin itself and it could damage the surroundings.

And these are the questions that we struggled with. How much of these oxidized materials do we allow in the patient and what will they really do. All of these questions we have asked again, manufacturers come to you with set of oxygen and equilibrium data and you were supposed to sort of, you know, trust these figure which they should do because if you look at the oxygen equilibrium curves and this is [pointing to the red cell curve] really everybody wants to have a curve very similar to the red cell but they come in variety of shapes and forms. Some of these hemoglobin are right shifted, some of these hemoglobin are left shifted, some of them don't even look curved. Are we supposed to just take these values at face value and trust that they will deliver oxygen ultimately to the tissue?

So these are some of the questions that we've asked at the time. Can in-vitro oxidation, the curve I showed you, do they really, can we use them to predict the tissue oxygenation? How about the oxidation reactions? Remember this is free hemoglobin. This is not inside the red cell anymore.

How can we control oxidation? How much is too much of the methemoglobin (oxidized) [that] we could allow? What about the safety, once the hemoglobin is oxidized? And of course more importantly, what about the role of normal endogenous-reducing agents that normally reduce hemoglobin back in the plasma? All of these questions needed, at the time, to be addressed. Recently we sort of come across one useful biomarker that we developed; this known as Hypoxia Inducible Factor (HIF).

This factor was discovered almost 12 years ago by Gregg Semenza at [Johns] Hopkins. It's like an oxygen sensor and, of course, it's found in every cell. So during normal, during oxygen normal level they have would in case of sufficient oxygen supplies, HIF would undergo [the] normal degradation process. In every cell there are enzymes that will tag HIF and it will be broken down under normal oxygen conditions when oxygen supplies are sufficient. However, when hypoxia sets in, HIF-1 will combine with its natural component, the beta subunit, and translocated to the nucleus, binds to the DNA and, of course, it will trigger a number of genes, including erythropoietin, a number of enzymes, proteins that control metabolism and oxidative stress enzymes and so on and so forth.

So the question is what if we have [an] oxygen-carrier carrying protein and depending on how much oxygen we carry, could we tip the balance? Does the oxygen carrier, hemoglobin, communicate with the oxygen sensor? Can we use the oxygen sensor to match it with the oxygen equilibrium curve and will it be able to have a very useful meaningful and productive tool in our hands? Here's an experiment in animal and this is a typical experiment done by manufacturers, about 50%, 80% of the animal blood will be taken out,

the HBOC will be infused and of course the idea of the HBOC will, of course, provide both the oxygen and volume. And here we've chosen two species of animals, the rat and the guinea pig, knowing before hand that the rat has the ability to produce ascorbic acid.

Ascorbic acid is a very powerful reducing agent. It will reduce the oxidation or control the oxidation of hemoglobin. The guinea pig, very similar to humans unfortunately, we do not obviously have the abilty to produce ascorbic acid, so we'll have to rely on nutritional uptake. for that purpose. So we infused these two animals with the same product which is approved incidentally, by the FDA for use in animals called, Oxyglobin produced by one of the manufacturers. And here you can see the rat clearly maintains sufficient amounts of ascorbic acid in circulation because of its enzymatic machinery. The guinea pig of course (Ascorbic acid) dropped to almost zero.

And when you infused the hemoglobin you can see the rat was able to control the oxidation of the hemoglobin but unfortunately the guinea pig of course could not do it. Now this is ironically very similar to actually what happens in humans. So now we wanted to know the level of oxygen sensing, how do [the] two communicate? This oxygen carrier, is it really delivering oxygen under these circumstances? We've chosen the rat. The rat is obviously cleaner, because it controls the oxidation so we should be able to actually look at tissue oxygenation without the complication of oxidation. And here, we're showing you, the HIH, the protein in the kidneys of these animals, non-treated and of course when you treat the rat with hetastarch, which is non-oxygen carrier, you can see the protein has been expressed.

Because the protein needs to be expressed for all the genes to be on to make up for the loss in oxygen supplies, more red cells are formed and so on and so forth. In the case of the blood substitute, there is some suppression of HIF, which you should expect that. There's oxygen at least being delivered in the early hours. And of course later on, the oxygen supply somehow got [a] little bit reduced and, of course, that we know because of the oxidation of the hemoglobin, even in the case of rats. Here, we're looking at the EPO, the gene and the protein and the plasma and, as you can see, in these animals, when you give them the starch, EPO increased in the amount, 30-fold because you're not giving an oxygen carrier.

When you give the blood substitute, actually, there is some delivery of oxygen at least, the first eight or nine hours. And remember this is really the golden hour so to speak because this is where the situation where a patient will need it, a patient who has lost a lot of blood and if you can bridge the patient until you get to the point of care, you basically achieved or accomplished your target. Here is, of course, the level of EPO in plasma, and the protein as they match the gene and the protein match nicely. Here's the problem, after a few hours, the hemoglobin is out of control, oxidized, and the heme possibly has been lost. We don't really know what happens after the eight hours or so. So that's one problem.

What we're trying to do now, that's not really the end of this story, what we're trying to sort of perfect the understanding of this biomarker to a point where we, before even we start with the animal studies, will have at least some good idea when somebody comes to me and gives me a hemoglobin with a P50 of so much and with the right fine tuning and all the properties, I

will be able to at least have some ability to predict tissue oxidation with these markers. So this is not really, the story is not, hasn't ended, we're still working on it.

And this is the big one. The toxicity, and that's really what's killed the field basically. Because of the heme and if the heme is going to only just come out of the hemoglobin, it could do a lot of damage even before it even comes out of the hemoglobin. So we have spent a lot of time and energy to develop some mechanisms whereby we can minimize the damage and thus we can control some of these side effects of hemoglobin. If you look in the literature and look at some of the side effects, well documented side effects in animals and of course in human, this is really what you see… it basically includes, hypertension and this is well known, phenomena whereby if you had given an animal or a human these HBOCs, immediately blood pressure would go up.

And due to constriction of the blood vessel and I will show you that because of [a] very simple reaction… and the rest of the list is really related to this very early reaction of course and the heme itself. Enzymes go up, pancreatic effects, oxidative stress, cardiac effects and the list goes on and on. The site of the problem is really summarized in this cartoon, which is in simple form. This is the blood vessel and what you're looking at basically, the wall of the blood vessel, which is, you know, is lined with these endothelial cells that produce nitric oxide (N.O.) and I'm sure you've all heard about this small diatomic gas that we actually produce and it has tremendous functions, signaling, and many variety of physiological functions.

Now we produce nitric oxide, nitric oxide has a very short half-life, almost to two seconds. But you can see even in this short half-life, it actually covered quite considerable distance. It actually reaches to the RBCs, to the red cells when they flow by the blood vessel wall. As they flow, they create an area which is known as RBC free zone. The red cells would not hang around near the vessel wall and leave that little space. When you have free hemoglobin, when you have a blood substitute, the story will be different. Remember the hemoglobin is very small; it's not going to flow with the red cell. It's going to come close enough to the wall

Hemoglobin has a tremendous ability or affinity to react with nitric oxide. And in fact, the affinity of hemoglobin to bind nitric oxide [is] almost 500 times more than the affinity of hemoglobin to oxygen, which is, its natural partner. So what happens, hemoglobin will react with nitric oxide, suck up nitric oxide, [the] blood vessel will basically constricts because "N.O." normally vasodilate [the] blood vessel, and of course the reaction creates an imbalance where "N.O." is a good guy and of course you have all these oxygen radicals, are the bad guys. So what you create here in this very region an oxidative mess, where the hemoglobin ultimately will break up, heme is released and that would initiate localized and possibly global toxicity or at least the beginning of the toxicity.

As an example, just to show you the power of the heme, here again, we run back to the same animals, the rat, and the guinea pig. We give them the same hemoglobin but this time we're looking at toxicity. And what we're looking at here are markers that we developed, which are activated normally when you occasionally break up your red cells, your heme oxygenase

enzyme which is designed really to mop up the heme and degrade the heme to produce bilirubin and what have you. And also heme oxygenase is used as a stress protein or used a stress biomarker. And as you can see here, after we infused the hemoglobin and again, in [the] rat because it controls the oxidation, [there is] very little heme loss; heme oxygenase's activity in the kidney was reasonable but look at the guinea pig.

If you look at the tissue here, we recently developed some tissue biomarkers and tissue toxicity. And here we're looking at oxidation of proteins in the tissues and we know roughly what sort of proteins we are dealing with because of the oxidation that [was] initiated or generated by the hemoglobin and you see clearly the damage and toxicity. How long is this damage? Is it transient? Does it have more consequences? We don't really know that but at least this point explains or at least demonstrates the sort of immediate changes, biochemical and cellular, changes, if you like within the tissue when hemoglobin is infused.

This is just to show you again recently we showed that even we see hemoglobin in the urine, we picked up the hemoglobin in urine and we've asked the question, does the hemoglobin actually undergo those changes because of all of these oxygen radicals? And truly we did find that the protein does undergo changes after it's been gone through circulation tissue and clearing the kidney, here we're using not blood substitutes, here we're using free hemoglobin, just to illustrate the point. And you can see changes in the protein and you can see actual changes at the amino acid level. Okay, so how we control it [toxicity]? What did we do?

Well, people actually started realizing years ago, we can't help it, the heme is toxic, it does deliver oxygen but you have to control the heme. So everybody is sort of running around trying to come up with a way to control this. Remember, people have tried fluorocarbons and many other forms of oxygen carriers. No protein really can match the hemoglobin. So we do have the ideal oxygen carrier. But the problem is either how can you control it, or how can you minimize the toxicity and get the maximum benefit? So we did like others; people resorted to reengineering the hemoglobin altogether, using recombinant technology and people basically focused on the heme and what they do in these approaches, they alter the heme environment, changing a couple of amino acids to make the hemoglobin less amenable to react with nitric oxide, less amenable to react with oxygen radicals.

They succeeded in the test tubes, and there are some good animal data, even one manufacturer actually produced newly engineered hemoglobin and things didn't pan out, unfortunately. We and many other people started looking at the naturally occurring hemoglobin because, as you know, nature has a wonderful variety of microorganism/ animals that live in variety of environments. One of these interesting stories a few years ago, we even looked into the clam hemoglobin from Puerto Rico. And this is the only free hemoglobin; one or two free hemoglobins in nature doesn't exist inside the red blood cell. It's actually free.

And when we looked at the heme, it has incredible ability to block nitric oxide and block hydrogen peroxide. These are the two elements that we're trying to sort of minimize their reactions with hemoglobin. The idea is not we're

going to go and find hemoglobin but the idea is to pick up a couple of clever ideas and obviously introduce it in properly engineered hemoglobin. Other people have tried, basically going back to the traditional thing, going back to the enzymes that exist in the red cells and take them out and of course add them to the hemoglobin using other antioxidant enzymes and ascorbic acid.

We have, recently with colleagues in Switzerland, actually explored another naturally occurring antioxidant in circulation. And that is haptoglobin (HP), and I'm sure you're all familiar with this very old protein, and nature had designed this protein to basically do exactly what we want to accomplish, to inactivate the hemoglobin in terms of oxidative changes. Here is a cartoon just to show you what normally haptoglobin does. Haptoglobin is of course designed to pick up the dimer not the tetrmer of hemoglobin. And when you occasionally, your red cells age and you release hemoglobin, haptoglobin, which is another protein that has alpha and beta chains.

It will basically bind to the hemoglobin, very effectively and through a receptor, hemoglobin and the haptoglobin will be picked up by the macropahges, it will be degraded, heme oxygenase enzyme and will de-activate hemoglobin. We even tried some chemically modified hemoglobin by changing some of the surface chemistry to make haptoglobin bind to the polymerized hemoglobin. Here is another example which we have just done, just to show you actually, well, we were surprised while we were working on this reaction. Paul here and Dominik in Switzerland actually discovered very interesting additional property of haptoglobin and that is it can lower the blood pressure.

And that to us, remember this is what we were looking for, can we control hemoglobin oxidation, oxygen toxicity and can we also control the blood pressure elevation due to nitric oxide? And this is something that we have done recently; it's been published in JCI just last week, or actually been accepted last week. Here is the story. What they did, they took a dog known to have a lot of haptoglobin but they also induced the haptoglobin chemically by drug to produce more haptoglobin and we infused the free hemoglobin in these animals. This is the level of heme or hemoglobin and the heme concentration before and after treatment.

And here you're looking at the blood pressure, the arteral blood pressure. In those animals, the dogs would have had [a] high level of haptoglobin, blood pressure is almost normalized and those dogs without haptoglobin or sufficient haptoglobin, blood pressure goes up. And if you look at the urine of these animals, the control, you can see the urine totally red, because the hemoglobin is cleared by the kidneys very quickly. There isn't enough haptoglobin to complex it. And of course, the one with haptoglobin urine, as you can see very clear, that's in the dog. Then they infused the guinea pig and again, as you can see, the guinea pig when it's given starch, no blood pressure.

When it's given free hemoglobin, you can see the immediate elevation of blood pressure and this is exactly what will happen in humans, very similar profile. The first 10 minutes the blood pressure goes up. Within a couple of hours, the blood pressure subsides. And of course the idea here, the clinical judgment is completely conflicted here. Some people say it is okay but others, blood pressure elevation in some people of course say no, we can't,

depending on the patient. But having said that and when you look at the data, haptoglobin clearly brought down the blood pressure. It also protected the tissue. Remember what I showed you earlier. The tissue toxicity.

When we looked in the kidney of the animals treated haptoglobin the toxicity almost disappeared. So that led us to sort of use our imagination and say well, can we actually use this as a new paradigm to engineer a new blood substitute? And that's what sort of led us to think and we are working on that, not necessarily we'll have haptoglobin, hemoglobin complexed as a blood substitute, this is really the beginning of what we are planning on doing. The complex would look something like this. The young lady sitting here from Switzerland actually did some of the imagining of this. This is the haptoglobin and the two subunits of hemoglobin.

And this is what we basically infused. What surprised us actually is that when we looked at the oxygen carrying ability of the complex, and this is well known of course in the literature, it actually carries oxygen. Our work that we did, we showed that heptoglobin does not block the heme. So haptoglobin blocks the vulnerable amino acids on the protein, prevents them from oxidation but leaves the heme exposed, as the heme of course is carrying oxygen. You can see that, not a lot of oxygen though. And more importantly, nothing happened to the hemoglobin structurally. It looked healthy as we would normally, as experts, we can tell.

And actually interesting, it almost like binds nitric oxide and it's natural because the heme is still available so it binds nitrogen oxide, good or bad, that's debatable. It carries oxygen. It circulates longer in blood. It reacts

with nitric oxide (NO), no blood pressure elevation and of course it's more stable in circulation. So we thought maybe we can sort of shift the focus from chemical engineering and genetic engineering to something actually simpler. It can carry the oxygen, haptoglobin, but it would not reject haptoglobin because it's there. It's actually new since because we work with the plasma derived product, we derived [unint.] the enzymes from the blood haptoglobin there in the larger quantities not a lot, but you can engineer it.

The idea here, what we're trying to say is we can shift the focus and we can start thinking maybe in a completely new way. So what we did in terms of our research successfully and not terribly successfully at least this is what we did for the last 20 years. We have sort of established methodologies to understand the fact of these chemistries that manufacturers put in their product and their effects on the stability, toxicity of the hemoglobin, we developed a number of cell in animal based models and developed [a] number of markers and we even started to think, we don't really know if this is going to pan out or not but at least we started to shift the focus and maybe that can be an [unint.] that we can play around with it in the future.

The people who actually did the work are all listed here, all of them from CBER and my lab. Dom and Jen sitting here, Paul is here, the pharmacologist, [unint.], Felicie is not here. Wayne, Elena, Dom is here and Francine. Dominik from Switzerland and Claudia are here. Thank you very much.

Q&A

FS: Thank you. That was great. There is time for questions. I'll kick off with the first one about why that's a different biological outcome.

A: We think, the haptoglobin and hemoglobin complex because it's a large complex and because it's probably large on the properties, such of the separation of the hemoglobin from the vessel wall. Remember, when I said nitric oxide is produced by the vessel wall and how it has really a short half-life and it doesn't really travel much. So the haptoglobin and hemoglobin complex, we think, is doing what the red cell is doing, keeping itself away from the vessel wall. But it still reacts with nitric oxide if real nitric oxide gets to it. And in this case we shouldn't worry about it because the blood pressure didn't go up because we separated the hemoglobin from the vessel wall basically.

FS: Other questions?

Q: [Inaud.].

A: Say that again. I didn't follow the first part.

Q: [Inaud.].

A: No. Well, we were busy with the hemoglobin actually. But your point is absolutely right because what you're doing there, something triggers the nitric oxide sensors and producing sufficient amount you get hypertension. So something in these products must have triggered the vascular effects activated something like you said rather or what have you, that produced nitrogen oxide. We do, I mean, if you talk about measurement in animals. We are set up to measure the salt level and hopefully once we get our new machine, we'll be able to measure nitrite and these other byproduct in the animal because we need it for this particular study on all those so we will be able to measure NO in cell culture, we do that, but in animal very shortly.

Q: [Inaud.]

A: We never really did [unint.] together and we're hoping and if our sort of imagination if you take it to the logical level, we have separated the complex

from the vessel walls. So in theory, we're creating a situation whereby nitric oxide reaching that complex in a minimal amount. See what I mean? We're creating a situation very similar to the red cell. But you're right. The data in-vitro. These are, we're looking at the oxygen binding separately from nitric oxide. Nothing will stop the complex from competing with the two, i.e., if you have a very similar situation where you have an oxygen carrier and a NO, there will be some competition between the two gases. But what we're hoping is that because of the physical separation from the vessel wall, we're at least minimizing them the contact with the heme.

Q: [Inaud.].

A: I know, I remember, we actually worked on iron.

Q: We did. I was curious though, is there new testing that's done for hemoglobin substitutes that in terms of possible [inaud.].

A: Yeah, actually, we did that and we published few years ago. When you put the free hemoglobin or HBOC with platelets nothing happens. But when you activate the platelets, start producing nitric oxide and other oxygen radicals, then you will see things go a little bit out of control. Animal work, we haven't done it but Barbara Alving one of the early FDA hematologist now at the NIH, she did in her dog model or some other model, I don't believe Paul would know this, she looked at platelet activity and blood substitute and some people say, yes, she sees some affect on platelets levels and activity. But it sounds, as I said, when in-vitro in our simple system, the two together no activation, no stress, nothing happens. But obviously when you activate the platelets things would be different.