UNITED STATES FOOD AND DRUG ADMINISTRATION

RED BLOOD CELL WORKSHOP

PRE-CLINICAL EVALUATION OF RED BLOOD CELLS FOR TRANSFUSION

Bethesda, Maryland

Friday, October 7, 2016

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Introduction:

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SESSION 5 - Potential Mechanisms of Red Blood Cell Transfusion Associated Toxicity

Toxicity of Acellular and Cellular Hemoglobins:

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Role of Nitric Oxide and Free Heme in Mediating RBC Transfusion Toxicity:

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Effect of Transfused RBC Storage Age on Outcome in Infected Canines:

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PARTICIPANTS (CONT'D):

Coagulation Changes Related to RBC Transfusion:

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Microparticle Medicated Toxicity:

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Transfusion Related Immune Modulation (TRIM)
Safety Issue:

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PARTICIPANTS (CONT'D):

Panel Discussion Leader:

PAUL BUEHLER

SESSION 6 - Summary of Panel Discussions with Gaps Identified and Future Directions Presented by the Panel Leaders

Determination of Suitability of RBCs for Transfusion:

PHILIP C. SPINELLA

Methods for Detection of RBC Processing and Storage Lesions:

THOMAS J. RAIFE

Animal Models - Oxygen Delivery and Perfusion:

HARVEY G. KLEIN

Animal Models - Shock/Trauma Resuscitation:

PHILIP C. SPINELLA

Potential Mechanisms of RBC Transfusion Associated Toxicity:

PAUL BUEHLER

Final Thoughts:

PAUL M. NESS, MD
Director, Division of Transfusion Medicine
Professor of Pathology, Medicine and Oncology
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Other Attendees:

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PARTICIPANTS (CONT'D):

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ROBERT ALLISON

DAVID ASHER

HELEN AWATEFE

JIN HYEN BAEK

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CELSO BIANCO

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P R O C E E D I N G S

(8:58 a.m.)

DR. BUEHLER: If everyone can take their seats. Our first presenter for this session, which is the Potential Mechanisms of Red Blood Cell Transfusion Associated Toxicity will be Dr. Abdu Alayash. He's from the Laboratory of Biochemistry and Vascular Biology. He's the lab chief. And he's my boss, so please welcome Abdu.

DR. ALAYASH: Paul, thank you. And thank you everybody for coming so early.

My task today is basically to really focus or talk about our experience with the toxicity of acellular hemoglobin and cellular hemoglobin. And that largely was built over the years on our experience with free hemoglobin, such as the blood substitutes, hemoglobin-based oxygen carriers. Then I'll do that. Then I'll see if we could actually touch on several issues that may be related to the storage lesion.

So the areas that I'm going to cover are really specifically oxidative pathways. And

again, this came over a number of years of experience with HBOCs and we've learned a lot from there, and we can apply it in recent years. I applied it to other conditions where hemoglobin is free, such as hemolytic anemia, in particular, sickle cell anemia. And there, of course, you have the situation where you have free hemoglobin and you have hemoglobin encapsulated in microparticles, which is a way of transforming or transporting hemoglobin to other vascular bed. And see how we can relate it to the storage lesion.

ETCHbox, or blood substitutes have been with us for almost three decades. They basically derive -- hemoglobin derived from outdated human blood or bovine, and they come in different sizes and shapes and forms because they modify them either by crosslinking the tetramer to stabilize it in tetramer form of decorating the surface of the protein, or polarize it, or even encapsulate it. All these chemical modifications or genetic modifications serve basically one of two purposes

-- to keep the hemoglobin tetramer stable and to prolong the circulation -- to prolong the half-life in circulation of the hemoglobin. That, like I said, provided us really with a unique look at how hemoglobin behave in a free environment, and we've learned, as I said, we've learned a lot from that.

If you look at -- so this is a very genetic list of some of the adverse events, and as you know, I don't have to tell you that after three decades of research and development, we don't offer till we have our product yet. And primarily because of the safety profile. And this is a list, a very generic list of some of the issues that have -- that we have dealt with in the community at large from hypertension to GI effect, pancreatic enzyme abnormalities, myocardial infarction, renal, and mortality.

And of course, you could ask some of the
(inaudible). They tell you so many
different mechanisms are involved
in here and we were preoccupied

over the years as to really what drives the toxicity of hemoglobin. From my perspective, there is one common element in there; it's heme-mediated events. Hemoglobin is called an honorary enzyme. Hemoglobin can actually interact with so many molecules. Just leave hemoglobin to its own devices. It will do all these enzymatic things for you. So the common element, as far as I can see, is really heme-mediated interactions.

This is -- over the years again we've sort of learned a lot. So this is just to sort of summarize the way we see it as far as the oxidative pathway. And also, how we in normal physiology, how do we deal with free hemoglobin and

(inaudible) due to hemolysis, aging of the RBCs and so on and so forth. And as you are all aware, we are

well-equipped to deal with small quantities of hemoglobin. And this starts really from the genesis of hemoglobin. Early erythroid cells where you're putting together the hemoglobin. Alpha subunit is unstable, and if you leave alpha subunit in a solution with beta, it will damage the beta subunit. So nature had provided AHSB, alpha hemoglobin stabilizing protein. It's a small chaperone of protein. It basically locks the alpha subunit in a form that it would not produce any radical, would not receive any electrons, and so on and so forth.

So that's the early genesis. And of course, when beta subunit comes in to make up the protein, AHSB disappears and you have a tetramer in circulation, and the RBCs, as you know, we have very sophisticated enzymatic machinery, primarily

to keep the hemoglobin and the iron, of course, in the ferrous forms. And even though occasionally -- or not occasionally, when RBCs age, we do have -- we tend to have a little bit more of the ferric form, one to two percent, which is okay. All these things will change when hemoglobin hits the free environment, circulation, be it hemolysis or in our case, ETCHbox where you're infusing considerable quantities of the protein.

What we have seen, based on our experience, once you leave hemoglobin in a free environment, it will kick in its own self-destructive pathway. There are some changes that ultimately lose the heme, and the heme now we know is actually people started to consider it as a damage associate, more like a pattern of dam, and I will explain that a little bit later on.

But again, nature had provided us with all of these interventions. Haptoglobin as we have shown recently on many other papers and work by Paul, it can actually stop hemoglobin's destructive path in a very clever way of doing it.

And if that fails, you have the hemopexin, you have A1M, and you have albumen. You have so many proteins in circulation that can take care of the hemoglobin.

When you overload the system -- hemolysis or in the case of ETCHbox, things will be a little bit out of control.

What just happened to the hemoglobin? This is what we spent a great matter of time to really understand this destructive pathway. Hemoglobin, as you know, can produce its own radical, rather oxidant. It can interact with oxidants. It can transform itself from a harmless, ferrous form to -- oh, sorry -- to a highly reactive form, which is the ferile form here. And also a radical. What we discovered is in the process --

Hello. What is this? Did I do something here? Good morning, sir.

Okay. So where were we? So what happens when the hemoglobin starts

generating oxygen and it goes into all

these forms, we find out that the targets of it is actually the beta subunit. More specifically, a handful of amino acids are clustered there in what we call the "hot spot" region, particularly cysteine 93, and keep that in mind. Once you hit cysteine 93, the beta subunit collapses, alpha subunit collapses, heme is lost, and so on and so forth. So at least this is what we think what actually happens when hemoglobin is left to its own devices. Add fuel to it, the reaction will be even more faster.

So in recent years we've done this experiment with Greg Vercellotti, in Greg Vercellotti's lab, and they have the dorsal skinfold chamber, which is less sophisticated than (inaudible). But it did serve a purpose. What they do, this is a sickle cell mouse and they basically look at the stasis, which is the vaso-occlusion, and as you know, they count that and as you know, this is a whole mark of

sickle cell of the sickling process. What we want to do in here is a very simple process. We're giving hemoglobin in different redox sites and we're giving heme to see what happens to that sickle cell mark. And remember, events in the marks are a little bit more exaggerated, so this is obviously we wanted to see that.

So this is an example from the work that we've done that was published. Here we're given a hemoglobin ferrous and you can look at the degree of vaso-occlusion or stasis. As you can see, when you add haptoglobin or even hemopexin, very effectively they reduce the level of vaso-occlusion. If you give the animal the heme, of course, you can get a considerable increase in the vaso-occlusion. If you add hemopexin, it will take it away. We even infused the animal methemoglobin. One hundred percent methemoglobin.

It did more or less the same degree of damage. When you block the iron with cyanide, it's not going to do much to you.

The lots of experiment besides the skin organ in this animal that Greg Vercellotti and John actually did, this really summarizes what we have reported that basically heme released because of these oxidative pathways that I've described, cellular or something along those lines. Heme is lost -- heme, now we know, binds to TLR4, and TLR4 is a well- established path for their inflammation and ultimately vaso- occlusion. And we think now we actually mapped out, not fully, the path to the inflammation which we've seen in a number of situations.

How do we -- what did we learn? How can we relay that to storage lesions? This is a slide that was kindly given to me by Franklin Bunn, who we actually invited but unfortunately couldn't make it. Or he didn't respond anyway. He didn't make it -- that he presented at a 2008 workshop along the same lines, the HHS organizers workshop

at the FDA. But this is what he defined then, and I think it's applicable. These are the issues related to the lesion from deformability, structural changes, impaired flow, oxygen, and more or less, this is more really related to what I've been describing where you have a considerable amount of free hemoglobin in circulation after infusion of an old bag. And of course, NO metabolism.

So in recent years, in fact, Paul's program, started in our shop, we started thinking of the red cell aging and the lesion. And this is the sort of proposed pathway that we sort of came up with. And based on some anecdotal literature data, which says basically, freshly minted RBCs, you've got the active enzymatic machinery there, but with time, age, or disease in the case of sickle cell, the anti-oxidative machine is more centralized in the cytosol, and that would lead in our thinking to sort of hemoglobin escape from the reductive machinery. Through the membrane you have lipids and these are the source of reaction

I've described that actually occur there which lead ultimately to band three clustering. Of course, you can get the physical formation or the microparticle formation.

In the lesion here we have an additional path you have to worry about is the hemoglobin within the microparticles. This is another venue that you can actually transport hemoglobin to a vascular bed.

In recent years -- so we looked at sickle cell again because it's easy and the events are more exaggerated as I said earlier. Here you're actually looking at some course of changes of hemoglobin within microparticles. These are particles that we generate or circulating microparticles from the animals. And you can see this is the cleanest spectra. You can actually (inaudible) hemoglobin in a crude preparation such as a microparticle. So we can see the hemoglobin. We can clean it up, and we can follow the kinetics. And we even actually look at the heme peak and the HBLC and we look at the individual

subunit from time zero to time, let's say, 42 hours or a day or two, so on and so forth. So we can see what happens inside the microparticle.

And here is the kinetics of the microparticles again. Remember, this is sickle cell. What you see here, when you look at the oxidation (inaudible), you start actually about 30-40 percent. We find the same thing of methemoglobin to start with and we find the same thing in sickle cell blood from patients from the NIH. But the interesting thing is something happens to the methemoglobin. It gets reduced for a bit, sort of (inaudible) inside the microparticle, then shoots up. If you take free hemoglobin, of course, it's going to straight-line nicely. There's nothing there. It's not been affected by whatever the enzyme in the microparticle. And here is, of course, the oxy going away.

And we wanted to see actually if fervile, we can capture fervile in the microparticle, and that's not an easy task. We

were able, actually, to do -- if you're looking at the spectra where it says fervile hemoglobin, we actually picked up the fervile hemoglobin. After a little bit tweaking of (inaudible) by adding oxide because we have to do that. So in other words, we can see these events. We're monitoring them from zero to the end, and we're trying to relate these events to what actually -- to the anti-reductive protein, the anti-oxidative enzyme. So with some initial proteomic analysis of the microparticle and with a focus -- sorry -- with a focus on these enzymes. And we can see actually there is -- this is early, in the early stages of the work, but you can see these enzymes, there's something happening. I don't really know. I think there are clearly changes happening in the reductive capacity of the microparticle which in parallel

(inaudible) with the oxidation of hemoglobin. So we think there's a link between the two, and these two events will trigger the heme

release and whatever changes in the microparticle.

So recently we started to think of a target, sensitive target that we can pick up that we can relate these changes to the cell. And specifically, the mitochondria. And here you're actually looking at a typical oxygen consumption rate. The experiment is very simple. You take endothelial cells you have with microparticles of free hemoglobin and what have you and you start measuring oxygen consumption rates using the Seahorse analyzer. This is a real-time assessment. And then you use -- the experiment starts by adding several inhibitors known to inhibit specific parts in the mitochondria. And the mitochondria will respond accordingly. When you add ATP inhibitor it will go down. When you add uncoupler, the oxygen consumption rate will go (inaudible), and when you add retinol, it will shut down the mitochondria. Your additive, regardless of what the additive was, hemoglobin or microparticle, it depends on the nature, we should

see if it's a healthy cell, it will respond accordingly. And as you can see, there's a little bit of a hint here that with the SS (inaudible) there's some retardation in the ability of oxygen consumption.

But when we added circulating microparticles, these microparticles being in circulation of the animal for a long time, obviously, things are happening over there. And you can see you almost reached a total shutdown in the mitochondria. We did heme oxygenized. We looked at the proactive oxygen species in the mitochondria, and we've even looked at the Band effect. And you can actually see that tyrosine-21 is being phosphatized. And this is the first sign of Band 3 clustering.

So to sum up, whether it's free hemoglobin or microparticle, if you have the same thing, all these events can occur which leads to obviously transition of hemoglobin into different forms. All of these events can be intercepted by

haptoglobin, hemopexin. If that fails, when you have overwhelmed the system, heme will always be released, bind to TLR4, and now we can have a target such as a mitochondria and you can actually assess all these events.

So this is the summary. And I see Paul start moving. It's basically what we're saying here to sum up is that oxidative will occur, whatever hemoglobin is free or even in red cells that are aged or diseased, and the target cysteine 93, you break up the beta subunit and that leads to heme release. Microparticles on the other hand provide you with another level of transforming or transporting the oxidized, damaged hemoglobin to a vascular bed.

Finally, this is another slide from Bunn, which is sort of his own obviously conclusion, which I would agree with the thought that really physical changes in the red cells in the microparticle are the issue that we need to deal with.

These are the people in the lab, who

work in the lab, and people I've collaborated with, working with

(inaudible), Jeff Miller, and

(inaudible). Thank you.

(Applause)

DR. BUEHLER: Thank you very much, Dr. Alayash for a very interesting presentation.

Next, we're going to hear a presentation from Dr. Rakesh Patel. He is a professor of Pathology at the University of Alabama-Birmingham. Also, the Director at the Center for Free Radical Biology Translational Sciences. And he will talk today about nitric oxide, as well as heme and its role as a potential toxic mediator in transfusion.

DR. PATEL: Thank you, Paul, and thank you to the organizers for not only inviting me but really putting together I think a much needed workshop that's, I think, hopefully will shed some clarity to a very complicated situation.

So what I'm going to do today is talk to you about some of our interests which over the last few years have centered on understanding what

happens to red cells when they're stored, what changes occur, and then trying to link them or ask the question of are those changes linked to any possible adverse effects in the recipient. And these questions have been articulated very clearly from people yesterday, and I'm sure will be further today. And they're quite simple questions. You know, is there a problem? If so, what is the nature of the problem and what can we do about it? I think it's fair to say is what we know is that the answers are not so simple and they're complicated, and they depend. They depend on really the exact nature of the changes, what's happening, and the recipient, the transfusion recipient.

So with that in mind, our focus has been on trauma patients. The reasons are outlined here. It's the leading cause of death in young individuals. The severity of injuries has been predicted to increase despite improvement in clinical care and management, and importantly, these patients consume a large portion of the

stored blood supply.

And I think also it's clear that there have been several retrospective analyses testing or asking questions regarding the storage lesion in trauma patients and most of them, if not all -- all but one, this was summarized in a paper by Rosemary Sparrow last year, show some association, positive association between either the amount or the age of the red cell and some adverse outcome in these trauma.

I'm just showing you one of these from some of the studies performed by collaborative (inaudible) Jordan Weinberg a few years ago where they looked at about over a 10-year span incorporating about 1,600 trauma patients who received exclusively old or fresh arbitrarily assigned. Here old is more than 14 days. And in this table they labeled these patients based on young and old but who received similar amounts of red cells and found that there was an increased relative risk of pneumonia as a function of those patients that received exclusively old.

In addition to that, if you take the same group and look at risk for death, then there was a dose effect also, those that were transfused with one or two units relative to those that were transfused with one or two units. Those patients that received more than three units had a high risk of death.

So I think it's important to recognize that we have to underscore the fact that it's an age and an amount problem, potential problem, at least in the context of trauma. And that's important when it comes to defining the right model or an applicable model to test the questions that we're all interested in.

Following that up, more recently we've been focused on the massively transfused trauma patients. These are defined as those that receive 10 or more units in the first 24 hours and they often, or they do get the oldest red cell units also. So the thinking here is that this will hopefully provide the biggest experimental window, if you like, for us to observe a problem if there

is one.

So this, I'm going to show you a couple of pieces of data here. This shows that the odds ratio for mortality in these patients over -- this is a retrospective analysis again at UAB over a four-year period, increases in a dose-dependent manner. Obviously, this doesn't say anything about the age. To try to get at that question, we've done a subanalysis where we took these patients, these massively transfused patients and stratified them to -- those that received two-thirds of their red cells were young. We call those mostly fresh. And the other group was mostly stored. Those that a third of which received young, and a third of which were older, and the rest in between. And if you do things like that, you can show that there is an association, significant increase in the relative risk, the adjusted relative risk for death, 24-hour mortality, than those that received mostly stored.

I put this out there because I'm hand

waving a little bit because these studies, these are retrospective of the difficult -- there's a lot of caveats. And really just to underscore the fact that we need, I think, in this particular area, a controlled, randomized study. And this is, again, easy to say, much more difficult to implement. And I put this up there hopefully to foster discussion later on as to how best to do this. And I know we at UAB have been thinking about it. I'm sure others have as well, and maybe we can talk about that more later on.

So moving on, there have been newer studies that have also shown that old red cells -- a transfusion with older red cells is associated with risk in high-risk patients, and there are several studies from animal model studies that support this idea that if you transfuse stored red cells on the background of trauma, hemorrhage, and shock, there's adverse outcomes.

I'll just show you a few of those here. The important thing here and the interesting thing is this has been demonstrated in diverse models.

You have rats, dogs, mice, and so forth. So there seems to be somewhat of a uniformity there. One of the take-home messages from all those papers is that, at least with trauma in stored red cells, it follows the two-head concept. The idea that if you take aged red cells and you transfuse a healthy person, you don't see anything really happen. But on the background of trauma, hemorrhage, and shock, these individual, these animals are already under some stress and now you see this increased inflammation, microcellular dysfunction, and increased infection.

To illustrate that, here's just some examples from our lab. This is a mouse model, a C57 Black 6 mouse model where they undergo hemorrhage, about 60 percent blood loss over 30 minutes. Shock is maintained for an hour, and then we transfuse with leukoreduced red cells with varying ages and the units over another 30 minutes, and there are several outcomes you can measure. Here I'm just showing you changes in markers of acute lung injury, looking at the

levels of protein or inflammatory cells in the bronchoalveolar lavage fluid. Do you see here? This is the saline. This is the injury from the trauma hemorrhage itself. If you on top of that transfuse fresh red cells, no effect. If you transfuse stored red cells, that goes up.

And similarly, this is looking at mortality, so you see that those transfuse to saline. There's 100 percent survival. If you transfuse with five-day red cells, survival goes down a little bit, but if you age them a little bit further, only 10 percent of those animals survive. So again, storage-age dependence.

And interesting here, if you take these red cells and you wash them, so you remove all the supernatant fraction and you transfuse with those pelleted red cells, you reverse that mortality effect.

And that gives rise, coupled with a lot of these other studies with this general picture as we see it is that you have a continuum. You've got the healthy red cell and during storage lots

of things happen to the red cell, again, as has been demonstrated yesterday. You have changes in the red cell, red blood cell morphology. You have formation of microvesicles, free hemoglobin, heme, iron, and so forth. And I think it's quite interesting. As a field, we know quite a lot about this, but then we think of these in silos going down in terms of what could these products be doing in the context of transfusion and these adverse effects?

And just to summarize, these are just some of the key ones I think that have been shown. We know that aged red cells, the microparticles hemoglobin will inhibit NO bioavailability, that microparticles, hemoglobin, heme, iron, they all have mechanisms, all have been described to increase inflammation, oxidative stress, and increase the susceptibility to infection.

So I'm just going to show you some snapshots of data to underscore or highlight the issues with respect to NO and some of the inflammation and infection.

So with respect to NO, the central reaction is this one. NO reacts with deoxyhemoglobin or oxyhemoglobin to form nitrosyl-heme or nitrate (inaudible). And this reaction is very fast. Ten to the seven per mole per second when hemoglobin is in its free form. What we know is that when hemoglobin is within the red cell, this reaction is slowed by about a thousand fold. But when you have the transitions of these red cells to the smaller, more denser red cells, all the way to these microvesicles of cell free, those packaging issues are gone and now this reaction is very efficient at removing NO from the system.

This is just an illustration of that showing the rate of this reaction, the relative rate of hemoglobin savaging NO when it's packaged in the red cell relative to the free form. When it's in an intact healthy red cell it's down here, but if you store red cells and they look like this, you see that rate goes up. So that's just showing that this reaction is accelerated as you

just go from this step to here. And this would be even higher as you go all the way down to here.

The bottom line is there are many mechanisms with respect to NO, and I think Alan Doctor summarized this nicely yesterday. You have the NO scavenger. You have loss of (inaudible) substrates like nitrite. You have loss of ATP release. Loss of snow, possibly. And then inhibition -- or culminating in inhibition of NO signaling.

This is just demonstrated here by Mark Gladwin's group. I think this was shown yesterday as well, that if you transfuse with red cell supernatants from young red cells, you see a slight hypertensive effect which is exaggerated with supernatants from stored red cells.

This is just some data from our group showing in an ex vivo model of the aortic ring bioassay that adding red cells inhibits interdependent dilation that increases the

function of storage age. And this has been confirmed and reproduced by others, John Roback's group, who they and Mark's group have gone on to show that similar effects occur in the context of looking at a flow-mediated dilation in humans.

The correlate to that is that if you put NO back or replete NO, can you prevent some of the injury? And yes, you can. This is just an example using nitrite. I showed you this earlier. This is the increase in BAL neutrophils. You can reverse that with nitrite and you can reverse the mortality effect in that trauma hemorrhage model.

So let me shift gears a little bit and focus on this guy, free heme, which Abdu gave a nice introduction to. I think in the context of stored red cell, this is -- it's been studied as extensively as some of these other mediators. So let me show you some data from that.

And just to dovetail a little bit more from what Abdu said, there's been a resurgence I think in heme biochemistry or heme biology. We've known for many, many years that free heme is a

potent lipid peroxidation stimulator. It potently stimulates for several paroxetine types of reactions, but what's come about recently is this understanding that it can also activate inflammatory signaling, like TLR4 and others. These are just a couple of review articles that show that. The idea that these are erythrocyte DAMPs, mediators of sterile inflammation.

So we wanted to ask this question in the context of the storage lesion. And one of the first things was, does free heme increase during storage? It sounds like an easy question but it's a little bit more difficult to address. And I put this in there, this slide in this morning just to dovetail with Jason Acker's presentation yesterday which highlighted -- one of the question he was raising was about methods. There are several methods out there to measure heme, and I think the issue is that many of them or most of them that are used, it's basically by a commercial kit, are not selective or specific.

This is just an example. If you --

these are just two commercially available kits that are marketed as a hemoglobin kit or a heme kit. If you add heme, both kits pick that up. If you add hemoglobin, both kits pick that up equally. Just as demonstrated here, if you mix equal amounts of hemoglobin or heme, this is what you should see but you see the additive effect. So these kits, depending on which one you use and how you use it, are not going to be able to -- they'll measure both hemoglobin and heme with more or less equal sensitivities.

So fast forward, we've been using and developed a spectral discombobulation approach -- I don't have time to go into the method here -- and applied that to measuring heme levels in stored red cells.

So shown here is the oxyhemoglobin level, and in paired -- red cells that have been stored for 35 days in paired bag and segments, and consistent with previous reports, there's high levels in the segment compared to the paired bag. Interestingly, for free heme it was the opposite

trend in that the levels in the bag were higher. And what I find interesting here is that these levels are now not too far from the levels of hemoglobin in that bag.

So another question is when we assess hemolysis as a quality of storage, are we just measuring hemoglobin or actually -- and if so, are we underestimating the hemolysis? Because we're not accounting for the free heme fracture. I'll just throw that question out there from a discussion perspective.

And then we just, because we wanted to know, to get a framework in terms of concentrations and amounts that are being transfused into patients. So this shows you the data. This shows you the level of hemoglobin or heme, albeit a sample from segments in 25 patients who -- these are all trauma patients that have been transfused. This is the level of -- the total amount of hemoglobin or heme in those patients that were not massively transfused and these ones in the massively transfused. Again,

this is what we're putting in. And I was just staggered by the amounts. The total amount within a two to three hour resuscitation period, approaching millimole for hemoglobin and 300 micromole for free heme. I mean, just huge, huge amounts of these compounds that, you know, several years ago we would have thought would just be silly to even think about that that that could be happening.

The other question, and again, I want to thank Abdu for laying the groundwork here, is where is this free heme? Because heme is very hydrophobic. Is it really free in solution? We don't have an answer to this exactly but taking cues from the sickle cell literature there is this idea that it might be packaged in microvesicles. And these can be characterized -- this is the red cell -- this can be characterized into both microparticles and exosomes. These are about 100 to 200 nanometers and these are a little bit smaller.

So this shows you the concentrations of

hemoglobin or cell free heme in a red cell. And you see that there's about 8,000 times more hemoglobin than heme. If you look at the microparticles and exosomes -- and these are collected from stored red cell units -- they are similar, they're lower than the red cell but the ratio has come down dramatically. In other words, you can almost look at it as if heme is selectively packaged in these small microvesicles compared to the parent red cell. Why does that matter? Because put packaging into these microparticles or microvesicles may change where they may go and how the heme works.

This is just illustrated. This is an example of taking red cells and the PED derived microparticles and exosomes, adding these amounts of heme, okay, to endothelial cells and looking at hemoxin as one as a sense for heme, and you see that depending on how it's packaged, the activity of that heme is different. So if you couple that to the ideas that these vesicles may be targeted to the endothelium and so forth, you change the

nature of how that heme may be a pro- inflammatory agent.

To show that it is playing a role in terms of the disease endpoints -- this is going back to our trauma hemorrhage model showing here if you transfuse with, in this case, day 14 red cells, one unit, there's no mortality. If you transfuse with three units you see about a 20 percent mortality. So again, a dose and a time effect. And that's completely reversed by hemopexin, the heme savaging protein.

This is showing the levels of inflammatory cells. Again, hemopexin brings that down. And interestingly, if you inhibit TLR4 with TAK 242, or you use TLR4 knockout mice, you can attenuate that injury largely as well.

We've also started to look as infection as another endpoint. And this data I thought was very interesting. So here what we've done is in that model of transfusion, hemorrhage, and resuscitation, 48 hours afterwards we instill in the airways pseudomonas aeruginosa and look at in

this case survival post-package administration. If the animals had received fresh red cells 48 hours before, there's no mortality over this timeframe. But if they received the older red cells, you see they all die within 16 or so hours. Lung permeability goes up and the colony-forming units, the burden of bacteria goes up as well, suggesting either enemy is oppressive and pro-bacterial growth environment. And importantly, all of these endpoints were reversed by hemopexin, improved survival, decreased permeability, and decreased lung levels of the bacteria.

So what this says overall is that we have this continuum of products that presumably derive from each other and it raises the questions, are there an interactions? If so, are they additive or synergistic? And you can, you know, on paper at least come up with several ways that that could be. I'll just give you an example is that we know that these guys scavenger NO. These guys promote oxidative stress.

Endogenously, we know NO is an anti-oxidant. So if you remove this, this is going to be more pronounced. And you can draw this out with different mechanisms. So I think that says something that we're challenged with is to understand, what is the interaction between these distinct mediators in terms of some of the mechanisms?

And just in the last two slides, within the question of "what is in the bag?" And what I mean by that is what is in the bag that may be functional in terms of post-transfusion toxicity, both in terms of a gain of toxic or a loss of protective mechanism.

I'm going to show you two slides just to, again, hopefully raise some discussion. This is data showing an LCMS run from stored -- the supernatant or stored human red cells. And we found something interesting that I think Angelo has also observed now, is that we found a modification of the beta-93 cysteine residue modified to a dehydroalanine, which at day seven

is low and at day 35 goes up significantly. And you can ask, well, so what? Well, the interesting thing there is we've got this transition, cysteine to this dehydroalanine. We've converted a nucleophile to an electrophile. So we're transfusing with something that's now changed. It is still hemoglobin but it may have very different properties than we would predict, just based on the fact that we've changed it from a nucleophile to an electrophile. So what happens? Does that change pharmacokinetics? Does it change where it goes? Those are all unanswered questions. And I will note that we don't see this in the C57, in a mouse storage model.

And my final slide before I summarize is the idea that it's not just a gain -- these mediators that are formed that result in a gain of toxic function, but perhaps we're also losing protective effects that are present in fresh red cells. And I illustrate that with this enzyme peroxiredoxin-2 which emerges as the predominant and the most significant antioxidant in red cells

that deals with biological levels of peroxides in other reactive species. It's indicated here the way it works is that this enzyme, when it's exposed to hydrogen peroxide gets oxidized ultimately to a disulfide and then gets reduced back. So this cycle is critical for protecting red cells from hydrogen peroxide for example. And you can pick that up in a nonreducing gel is that you have a very low amount of hydrogen peroxide, 500 nanomolar. You see that the monomer moves up to the dimer and then with time this would come down.

I think what's really important here is that -- these are studies by Christine Winterbourn, who showed that this is operating in the circulation to protect potentially or to be a part of circulatory homeostasis in terms of the redux signaling perspective. So data, what she did was she took mice and injected them with LPS and showed that red cell peroxiredoxin-2 is oxidized and that this peroxide is coming from neutrophils. So the red cells are a buffer in

that sense. Not only is it protecting against red cell-derived hydrogen peroxide but from hydrogen peroxide coming from activated inflammatory cells in the circulation. So in that sense red cells would be an antioxidant.

If you look at what happens with stored red cells, you see that peroxiredoxin, if you take fresh, you get oxidation that slowly then comes back down. If you go to day

red cells, it can absorb that first hit but it doesn't recover, suggesting that we're transfusing with a product that may not be as able to protect -- you've (inaudible) protective mechanism.

This is my last slide. And just to summarize -- I won't belabor the point -- but we got several mediators. I think the relative importance of it is going to be dependent on the model, the protocol, and so forth, but one area that we need to start thinking more about is how they interact and can that help us in defining which -- what will be endpoints of injury and

potential strategies to target those. And then we should consider not only a gain of toxic like these, but also loss of protective mechanisms.

With that, I want to thank the contributors, predominately Joo-Yeun Oh, who has done most of the work I showed you, and a former student, Ryan Stapley. And our clinical colleagues, Jeff Kirby, Henry Wang, and Marissa Marquez, and Jean Francois Hardy as well.

Thank you.

(Applause)

DR. BUEHLER: Thank you, Dr. Patel, for a very stimulating and interesting discussion.

And now we have Dr. Charles Natanson, who will talk about the risk of transfused older red blood cells in critical illness.

DR. NATANSON: Good morning. I'm extremely thankful for the FDA for allowing me to speak today. I'm going to present a series of studies we did in an animal model with Harvey Klein, looking at the risks of transfused older red blood cells.

I have no conflicts. There are two mechanisms of injury that I'm going to focus on. One was developed by Mark Gladwin and Dr. Schechter here at NIH that stored red blood cells accumulate cell-free hemoglobin, and as our previous speaker said, it's associated with nitric oxide savaging and vasoconstriction.

The second one is the iron hypothesis that was developed by Spitalnik and Ho at Columbia, that iron is an essential nutrient and promotes bacterial growth and increases risk of infection.

Why do we need a preclinical model? There's over 100 million units that are transfused yearly worldwide. Fifteen million units in the United States, approximately, maybe a little bit less or more, are four to six weeks old and these units have been difficult to study.

So the first question is do older blood cells increase risk of transfusion in critically-ill canines? The model we used was staph aureus pneumonia, and these animals are

treated just like in an intensive care unit. They receive mechanical ventilation, sedation, antibiotics. They get cardiovascular support, and fluids and antibiotics, and all their treatments are titrated, like ICU patients, based on physiological parameters.

We challenged animals with staph aureus to produce a model that was for critical illness where if blood was going to do something we would just have to tip them over from a 50 percent mortality. We transfused universal-donor, commercially-available canine red blood cells that are sold on the market, and consistent with human-stored blood cells, greater than 60 percent of the canine red blood cells with chromium studies remain in the circulation after 24 hours, and less than one percent hemolysis in the storage bag.

We exchange transfused the animals four times a quarter of their blood volume, with either 42 or seven-day old stored red blood cells. We did this at 4 to 16 hours after bacterial

challenge, and if you account for ad mixture, this replaces about 70 percent of the animal's blood volume. And this was the result that we were quite surprised with. As you can see here, the animals that got the older blood in blue had a marked increase in mortality compared to the 30 percent mortality from the bacterial with the fresh blood.

And this is a measure of the ability to oxygenate, and you can see here that the animals that got the old blood were not able to oxygenate as well and they had an increased alveolar arterial gradient, and so they had worse lung injury.

Let's get back to the two factors that I said I was going to focus on during this talk, cell-free hemoglobin and iron levels; what happened to them in the model?

Shown here on top in blue is the cell-free hemoglobin levels in the individual animals and the bar is the mean. This is during the time of transfusion. You can see here that

every animal had a marked increase in cell-free hemoglobin levels, and this is after transfusion. So it represents in vivo hemolysis. And you can see the fresh blood. And there was no increase or no increase in cell-free hemoglobin.

Next question, is it vasoactive? This is an assay of plasma NO consumption capability. This old blood was markedly able to scavenge nitric oxide so it was vassal active compared to the fresh blood.

So transfuse older red blood cells, increase cell- free hemoglobin levels, mostly in reduced oxyhemoglobin state. And we actually measured that; that only about 10 percent is in that hemoglobin state. It's also reflected by the increase in plasma NO consumption capacity.

So this is non-transferrin bound iron levels, which represents excess iron than the body can normally handle. This is seven-day old blood. The red line is the normal level that you shouldn't see anything above it. And you can see at these box of whisker plots during transfusion

there is a marked increase in non-transferrin bound iron compared to fresh blood. I'm not showing you later time points, but I will for reasons you'll understand when I show you the later slides.

So in terms of this canine study, our findings indicate older red blood cells increase risks. In studies that I didn't show you that we've done meta-analysis, the RCTs really studied a really different hypothesis than the randomized control trials, and they really studied significantly fresher and younger blood than the observational studies. And we found in the observational studies the blood is actually significant older and there's an increase in mortality.

So consistent with observational studies in canines with pneumonia, older red blood cells increased mortality associated with hemolysis, increased release of cell-free hemoglobin, and increased release of iron.

So the next question we ask is, is the

volume of transfused older blood cells a critical factor? These were massive transfusions. If you only transfused a couple of units of red blood cells, would you find the same result?

No, the volume wasn't important. We found that whether you gave 10 to 12, 4 to 6, or 2 to 3 units equivalent in humans, that the effect was fairly similar.

So the next question we asked, was the mortality risk of transfuse altered by the presence and severity of infection? Did you need to have an infection to find these increased risks of older blood? And to our surprise, what you found here is without any bacterial, older blood produced no toxicity. If you just had a mild infection where there was no lethality, it was a minimal increase. But when you had a moderate infection with a 30 percent mortality, there was a marked increase. And if you produced a rapidly lethal model, there was no effect of the old blood compared to the fresh blood.

Now, I'm going to be showing you more

data later in terms of the blood pressures and the iron levels and the cell- free hemoglobin levels from these same experiments.

So in healthy controls, there was no significant risk of older transfused red blood cells. With moderate doses of bacteria, older RBCs increased the mortality risk. Risks of older RBCs are dependent on the presence and on the severity of infection.

So now I'm going to show you the blood pressures, the systemic pressures, and ask the question, are the systemic pressures associated with older RBCs, modified by the presence and severity of infection? And please remember, transfused older red blood cells release cell-free hemoglobin that scavenges nitric oxide causing vasoconstriction.

So this is a moderate dose of bacteria, and I'm having to show you the shock score because we give these animals pressers. So their blood pressures are all the same. But if you're here, if I say you're better in terms of shock score,

you're not on pressers and you're hypertensive. If you're here, you're on pressers and you're -- you're on pressers, you require large doses of pressers, and up here is during transfusion, and blue here is the 42-day-old blood. And so during transfusion, the older blood caused hypertension.

But look here. When we got to the point where they became septic at 24 and 48 hours, the old blood causes a very different effect. Now it causes hypotension. So transfused older stored blood during transfusion improves, but after 24 hours worse and shock.

Does older blood, transfused stored blood really have two different effects on systemic pressures during infection? This is some of the data I like most because it gets to mechanisms and it defines interactions and main effects, and I believe it then, we'll tell you -- give you what are the effects of iron and cell-free hemoglobin in this model?

But first, from that dose response, I'm only going to show you the controls over time.

Only animals transfused without bacterial challenges and look at the shock score over time. And in blue again is the old blood, and this improvement. Notice, the old blood at 4, 13, 16, 24 hours, it's a main effect. It always -- without bacteria, old blood always causes hypertension.

So without bacterial infection, transfusion of older blood causes a sustained increase in blood pressure.

Now I'm only going to show you the animals that got bacteria and the dose response, bacterial challenges and what happened to the shock score.

So here's before transfusion. And notice the blue and the yellow. There is some mild hypotension from the anesthesia, but here is the middle of transfusion, 75 percent transfused. And it's now pretty much with established infection early, older blood increases shock score regardless of the dose of bacteria -- 1, 1.25, 1.5. Okay? But then, when we move to 16 hours

when the animals are really very septic, now note a very different pattern. Now there seems to be a change in the shock score or worsening of shock with increasing doses of bacteria that's significantly worse with the older blood.

Later, with established infection after transfusion, older blood decreases the shock score more with increasing bacterial dose.

So now we come to 24 hours after, and now there's really a profound effect that's statistically significant. Still later with an established infection, older blood after transfusion decreases the shock score even more with increasing bacterial dose.

So there's an interaction between the age of transfused stored blood and the dose of bacterial challenge, and we're going to try to find out if either the cell-free hemoglobin or the iron has the same interaction that can explain this effect.

With bacterial infection, older blood after transfusion with time causes a fall in blood

pressure that is greater with increasing doses of bacteria. There are two possible mechanisms of injury associated with transfused older blood. Early, vasoconstriction that is independent of bacterial dose; later, a worsening of shock that is dependent on bacterial dose.

So now, does the severity of infection alter cell- free hemoglobin or iron levels? Can we find that either one of these will have the same pattern that we saw of the shock score to explain those results? Can these factors explain why older red blood cells early on raised mean arterial pressure independent of severity of infection and later on decrease mean arterial pressure dependent on the severity of infection?

And here you see the old blood, the cell-free hemoglobin levels, and you see it's a main effect. It's not affected by the dose, as the blood pressure wasn't affected in the animals without bacteria. And this can explain that this rise in blood pressure that is a main effect.

So independent of the severity of

infection, increased cell-free hemoglobin levels are found in similar amounts in the vascular space for days after transfusing older red blood cells.

Now, let's look at iron levels, non-transferrin bound iron. Here's the -- above here is the abnormal level, and just before we transfuse these animals, note that nobody has a rise, nor should they, in non-transferrin bound iron. But what happens after transfusion? Well, this is why I didn't show you. I only showed you the early iron levels. What you find here is this is the old blood and here is the new blood, and these are the increasing doses of bacteria. What happened to the iron levels?

So as the bacterial dose increases, iron levels fall faster with older blood. We found the same interaction that we found with infection and systemic pressures. So transfusion of older red blood cells are associated with higher iron levels and more rapid decline as bacterial challenge dose increases.

So to summarize this part of the talk,

hemolysis of older transfused red blood cells results in elevated cell-free hemoglobin levels over days. The cell-free hemoglobin levels are not altered by the presence or severity of infection. Cell-free hemoglobin is vasoconstrictive and at sites of injury, and we found some evidence for this in the model, can potentially cause additional ischemic damage, worsening lung injury, and mortality.

In addition, transfused older red blood cells hemolyze, releasing iron, an essential nutrient that can promote bacterial growth. In healthy controls, transfused older red blood cells produce iron levels that are very high without increasing risks. Iron released during pneumonia is associated with increased risks, and as the bacterial dose challenge gets greater, iron levels disappear faster.

So now the question comes, is it just shock that you needed or do you actually have to have an infection? So we then went to a shock model without infection. And what we did was we

bled animals half their blood volume. We maintained them that way for two-and-a-half hours, and then we retransfused them either fresh blood or 42-day-old blood. It's a reperfusion injury model.

And yes, in this model we got marked increases in non-transferrin bound iron with old blood. And yes, we got marked increases in cell-free hemoglobin levels with old blood. But we didn't get worse outcomes. The 42-day-old blood had a better outcome than the 7-day-old blood, showing to us that iron and cell-free hemoglobin are not necessarily toxic; it depends in what setting you place them for them to either have a beneficial or harmful effect.

So transfusion of older red blood cells during hemorrhagic refusion injury increased cell-free hemoglobin levels but did not worsen mortality. This was a significantly different effect than during infection.

So now you're saying, how can this be? What was the mechanism? Well, cell-free

hemoglobin released by the transfused older red blood cells scavenged the nitric oxide, reducing the levels of norepinephrine that were required to main blood pressure. So the animals that got the old blood didn't require any norepinephrine. And the decreased norepinephrine dosing resulted in significantly lower cardiac outputs, which decreased reprofusion and its intended injury. So by lowering the need for norepinephrine and decreasing the cardiac output, the old blood decreased the reprofusion injury and prevented this injury from occurring. All the animals that died from -- that got the fresh blood had this injury; the right middle lobe of the liver was infarcted.

So the next question we sort of addressed, because we said, okay, old blood has increased cell-free hemoglobin and has increased iron. Well, what if you just give iron alone and compare it to fresh blood? Will you reproduce the same thing? Can standard iron therapy alone increase risks in animals receiving bacterial

challenges?

So we looked at during pneumonia with acute mild anemia. We removed two units of blood, and then we transfused either fresh blood or we just gave iron therapy and volume replacement. And these are pretty stark results. Here's the transfusion. Both iron sucrose, which is an older type of iron that's cheaper that's used more commonly, and ferumoxytol, which is a new iron that supposedly is taken up by the macrophages, and because it's take up by the macrophages it's not supposed to be available to the bacteria and produced similar, significant increase in mortality. And this is just the daily required dose on a per weight basis that the animals can get a one-time dose per week as recommended in the package inserts for these two irons.

And here's the lung injury. It looks just like the old blood. They both produce worse lung injuries.

Now, I'll take a second to show this data. This is the non-transferrin bound iron

levels. And you can see iron sucrose here. When you're giving it, you get marked increases, okay, in non-transferrin bound iron levels that are still elevated at 24 and 48 hours. Now, the ferumoxytol is in blue, and as stated, I don't have evidence for this, but it does disappear and may go into the macrophages. But in 24 hours it's back, and it's significantly elevated compared to fresh blood. And it's similarly elevated to sucrose. And that's also true at 48 hours.

I don't think I'll have any trouble with this data with a group of transfusion people. They won't mind this kind of data. They'll just have a suspicion.

The pulmonary capillary wedge pressures were similar. The cardiac indexes were similar. And the hemoglobin levels were not significantly different. They are higher with the transfusion model. Probably this represents the ad mixture from the spleen which dogs do.

So transfusion versus iron therapy. Iron therapy pneumonia with mild acute anemia

versus transfusion, increased mortality, increased lung injury, increased iron levels. These risks were independent of the type of iron therapy, iron sucrose versus ferumoxytol. Transfusion of fresh blood is associated with less risk than iron therapy during mild anemia with pneumonia. Further, raising iron levels alone independent of cell-free hemoglobin during mild anemia with pneumonia can increase risks.

So I'm finally at conclusions. To summarize, animal models of older red blood cells. Older red blood cells are associated with increased hemolysis, release of cell-free hemoglobin, release of iron. In pneumonia, these are abnormalities associated with increased lung injury and mortality. The increase of risks of older blood exists even at commonly used transfusion volumes, two units.

The importance of the RBC storage lesion is critically dependent on the clinical setting and the age of the red blood cells. Older, transfused red blood cells do not exacerbate and

may improve some situations, such as hemorrhagic shock reprofusion injury. If transfusion risks are dependent upon the RBC storage age, as well as the presence of infection, then RCTs evaluating relatively fresh cells or mostly unaffected patients are unlikely to detect these effects.

But getting back to the purpose of this meeting, RBC quality, animal models could help with preclinical determination of stored red blood cell quality. For example, animals may have real relevance in determining safety of stored red blood cells in various clinical situations affected differentially or differently by RBCs of different ages. This, more than a simple laboratory determination would link biomarkers with clinical outcomes.

Thank you.

(Applause)

DR. BUECHLER: Extremely compelling talk. Thank you, Dr. Natanson.

Now, Dr. John Weisel will present coagulation changes related to RBC transfusion.

John is a professor of Cell and Developmental Biology at the Perelman School of Medicine at the University of Pennsylvania.

DR. WEISEL: Thank you. My title slide has as a background red blood cells in a blood clot. And I'm going to be telling you how this shape change occurs during the course of my talk.

I'm going to start with Virchow's Triad that you all know. These are the factors that influence the risk of thrombosis or can also be applied to bleeding. And of all the components of blood that we know about here, many of these are very well studied, but I think red blood cells are very likely the least studied in terms of their effect on thrombosis and bleeding.

So we know from clinical studies over many years, there is a lot of indirect evidence for the role of red blood cells in hemostasis and thrombosis. Anemic and thrombocythemic patients with bleeding can be successfully treated often by elevating the hematocrit. Abnormally high

hematocrit can predispose a patient to thrombotic disease. Also, we know that there are many red blood cell abnormalities that can predispose patients to premature thrombosis, such as sickle cell disease, beta thalassemia, and other diseases. There are also diseases that secondarily alter the properties of red blood cells that can result in thrombosis. And we know, for example, diabetes, hypertension, and various thrombotic diseases.

We also know an effect of red blood cells on hemostasis and thrombosis in terms of flow. At high shear rates, the red blood cells marginate the platelets and put them next to the endothelial cells where they're poised to act in terms of thrombosis, whereas under low shear rates that does not occur.

You also heard yesterday from several speakers about the exposure of phosphatidylserine on red blood cells in various pathological conditions or with storage. So phosphatidylserine is necessary for coagulation. The prothrombinase

complex is assembled on phosphatidylserine, and it's usually been supposed that this occurs on the platelet membrane, because when platelets are activated, there is a large exposure of phosphatidylserine. But it's been discovered in the last 10-15 years that there is significant exposure of phosphatidylserine on red blood cells as well, and this also occurs with storage of red blood cells, an increase, and even though it's a relatively small percentage of red blood cells that have -- that normally have phosphatidylserine exposed, because of the hematocrit, the volume of red blood cells, this is a very significant source for assembly of the prothrombinase complex.

We also heard some about microparticles. I'm not going to spend much time on this because the next talk is going to be about microparticles. But microparticles from red blood cells are generally prothrombotic for a number of reasons because of exposure of these various components in their membrane.

We also know that there are direct

interactions between red blood cells, especially under pathological conditions with the endothelium. There can be specific interactions between red blood cells and platelets through specific receptors that are shown here. And there's also interim receptors in the red blood cell membrane that combine fibrinogen, and this may be important for aggregation of red blood cells. All these factors may be important in terms of coagulation.

Next, I want to talk about clot contraction or retraction. Hematologists commonly call this retraction, but I'm using the term contraction because it's similar to what happens in most other cells in terms of the mechanism. So this is the shrinkage of the volume of the clot that requires platelets and fibrinogen. It's thought to be important in hemostasis for forming a better seal, for restoring flow past obstructive thrombide by reducing their obstructiveness, and in wound healing.

And as I mentioned, this process uses

the same components of the platelet that are present in most other cells. There are -- there's non-muscle myocin 2A inside the platelet and actin filaments that bind to the membrane of the platelet via talin and kindlin. They bind to an integrin, alpha 2b beta 3, that binds the fibrin outside the platelet. So you have transmission of force from the actinomycin inside the cell to the fibrin that's outside the cell.

If you have whole blood and it contracts, if you look at the outside of that clot, you see mostly a very dense network of fibrin and platelet aggregates. When you look inside the contracted clot, you see red blood cells that have been compressed by the force of contraction to form these very tightly packed array of polyhedral red blood cells. And because they're polyhedral erythrocytes, we've named these polyhedral erythrocytes.

So no matter what the mechanism for initiating clotting and contraction, these structures are formed and there is a -- you begin

with a homogeneous network of fibrin, platelets, and red blood cells, and during the process of contraction, these fibrin and platelets mostly end up on the outside. The red blood cells end up on the inside. And the red blood cells are compressed to form this very impermeable network of polyhedral red blood cells. So this is probably important in hemostasis, especially venous hemostasis, but on the other hand, it helps to prevent the permeation or diffusion of fibrinolytic drugs into a contracted clot.

So is this something special about clotting that makes the red blood cells change from being normal biconcave to being polyhedral? So if you just centrifuge whole blood, the red blood cells become the same polyhedral structure. So it's just the forces that are exerted by the platelets pulling on fibrin that makes them undergo the shape change.

So we've studied this process. We've developed a special chamber where we can put blood and watch the clotting process as a function of

time to study the kinetics of clotting quantitatively. And if you look at the process of clot contraction with red blood cells, the extent of contraction is considerably less and the kinetics are different. I'm not going to go into the details here, but the kinetics of contraction are altered by the presence of red blood cells.

We also looked at the effect of the stiffness of the red blood cells, first, by using ovalocytes. Llamas have, instead of human biconcave red blood cells, they have -- ovalocytes have about two to three times as much spectrum so they're much stiffer than our red blood cells. And again, the kinetics are affected. There's much less contraction that occurs with the stiffer red blood cells.

So we also looked at contraction in blood from sickle cell disease patients, and they also have reduced contraction as a result of the change in stiffness of their red blood cells.

In contrast, although there's less contraction with stiffer red blood cells, the red

blood cells also, in the presence of red blood cells, the platelets exert more force. So this is the contractile stress that the force that the platelets are pulling on the fibrin during the process of contraction and that force is considerably greater in the presence of red blood cells. So when the platelets are pulling against the red blood cells, they pull harder.

So, as you know, clots or thrombi are different in the arterial system versus the venous system, and so this -- we would expect the effect of contraction, the effect of red blood cells on contraction to be more significant in the venous system than the arterial system.

So we developed a model that's used for examining venous hemostasis in the mouse model. This is using the saphenous vein. So you use a 22 gauge needle to poke a hole in the saphenous vein, and then we looked at the clots formed. And here are some scanning electron micrographs to show the structure of these clots, and you can see a lot of these polyhedrocytes, and you also see areas that

have this dense mesh of fibrin and platelets with the polyhedrocytes inside. And when we quantified these images, the polyhedrocytes make up about 42 percent of the volume of these venous clots. And biconcave red blood cells are only about less than two percent of the volume.

We also looked at coronary artery thrombi. Cardiologists are now using catheters to aspirate thrombi in treatment of myocardial infarction. This is a colorized scanning electron micrograph. I chose this area specifically because we tend to think of arterial thrombi as being platelet rich, but there are quite a few red blood cells in different areas. There's also a lot of fibrin. This is like a cholesterol crystal.

And when we quantify these images, we see that actually, the majority of these arterial thrombi, the coronary artery thrombi, are fibrin next to platelets, but red blood cells make up about 10 percent of the volume, even though these are arterial thrombi.

And these are some examples taken from MI patients, and you see this very densely packed array of this polyhedral red blood cells. And this is a study from another lab in Europe where they found the same structures in coronary artery thrombi.

So we're now looking at thrombi taken -- a venous thrombi taken from patients and also pulmonary emboli. And we haven't quantified these images yet, but we can see the same sorts of polyhedral structures. So these are present not only in in vitro clots but they're present in in vivo clots, hemostatic clots, and in thrombi.

We've also begun a study taking blood from patients with ischemic stroke, and using this method that I showed, looking at the kinetics of contraction and the extent of contraction. And what you can see here is that these stroke patients have much lower extent of contraction than healthy subjects. And this may, in part, account for their clayical condition because one of the functions of clot contraction is to relieve

the obstruction that occurs in thrombosis. So this may be one reason, one of the problems with ischemic stroke.

So in conclusion, in flow, red blood cells cause margination of platelets. Red blood cell phosphatidylserine is responsible for significant prothrombin generation and coagulation. Red blood cells combine the platelets to each other and to the endothelium. In contracted clots, erythrocytes are compressed to be these close-packed polyhedrocytes, which forms a very impermeable structure, and they're present in thrombi, and they're also present in venous hemostatic clots, and they confer resistance to fibrinolysis by decreasing permeability.

So I'll just end by thanking the people in my lab who carried out this work, and thank you.

(Applause)

DR. BUEHLER: Thank you very much. Interesting talk.

We will now move to the effect of microparticles, Dr. Jennifer Muszynski, from Nationwide Hospital and The Ohio State University will talk further on microparticle mediated toxicity.

DR. MUSZYNSKI: Good morning. Before I begin, not unlike the other speakers this morning, you'll notice that Philip Norris and I have dovetailing interests in both transfusion-related immunomodulation and microparticles. So you'll hear a little bit about TRIM in this talk, and you'll hear a little bit more about microparticles in the next talk, and kind of consider us a package deal here for the next half an hour.

Speaking generally about microparticles -- and here I'll pause and say that when I use the term "microparticles" for this morning, I'm going to use that catch-all phrase that's going to include all of the extracellular vesicles, including microvesicles, the larger particles, and exosomes, those smaller particles. So in general,

we're talking about particles that are going to range in size from about 40 to 1,000 nanometers. Now, those found in RBC products are generally going to be around 300 to 400 nanometers on average.

And while these were once thought to be just cellular debris of little consequence, certainly, as we've already heard, there are emerging data over the past several years now that microparticles certainly may play important roles in cell-to-cell communication.

We know that red cell microparticles accumulate over time during storage. We've heard that already this morning. What we haven't heard, and this is alluding back to Dr. Acker's work that we heard a little bit about yesterday, but manufacturing method may influence the accumulation of microparticles in stored red cell products.

In terms of cells of origin, for the most part these are generally going to be red cell-derived microparticles, although there

certainly are also platelet drive microparticles and endothelial derived. In prestorage leukoreduced red cell products there are very few leukocyte- derived microparticles, although there are some reports of some monocyte-derived microparticles that accumulate over storage even in leukoreduced red cell products.

And as we'll talk about in the next slides, red cell product-derived microparticles can mediate toxicity via dysregulated coagulation, which you've heard about a little bit already; disordered vasoregulation; as well as immunomodulation.

In terms of coagulation, this will be a familiar story now after the last talk. Certainly, red cell-derived microparticles generally do exhibit pro=coagulant properties in preclinical models. This has been evaluated by thrombin generation, TEG, platelet aggregometry, and bleeding time in animal models.

The figures that you see are examples of thrombin generation on the left either in the

absence or presence of red cell-derived microparticles; in the right, showing a dose response of red cell microparticles and thrombin generation.

The mechanisms of this, we've heard the phosphatidylserine story already. Phosphatidylserine on the outside of microparticles can certainly serve as a nitus for initiation of the coagulation cascade. Microparticles from multiple cell types, including potentially red cells

(inaudible) tissue factor, and certainly, red cell-derived microparticles can also interact with platelets which have consequences likely for coagulation and inflammation both.

I've seen this figure before yesterday. Certainly, we've heard that red cell-derived microparticles do contain heme, which can effectively scavenge nitric oxide and result in a blunting of NO mediated vasodilation.

In terms of immune function, red cell-derived microparticles can interact with innate immune cells. This is a figure from the work of Dr. Norris's lab, and very nicely shows red cell-derived extracellular vesicles stained here in red, monocytes stained in green, and then the merge that you see after 24 hours in co-culture.

The effects of this interaction as reported in the literature are a bit mixed with both inflammatory and immunosuppressive effects being reported. And to illustrate that point, I'm going to go through some additional data from Dr. Norris's lab and some data from our own laboratory to kind of drive this home.

So again, this is a figure from Philip Norris's group. They looked at cytokine expression from PBNCs that had been exposed to extracellular vesicles from red cells that were stored for either 0, 21, or 42 days. And I'll call your attention over here to the extracellular vesicles from fresh red cells that resulted in

significant increases in pro- inflammatory cytokine production.

Now, in subsequent experiments, when they took monocytes that had been stimulated with extracellular vesicles across storage duration, I found that those monocytes then were capable of inducing T cell proliferation. So suggesting an overall pro-inflammatory phenotype from red cell-derived microparticles mediated by monocytes.

In our laboratory, we look at the monocytes themselves. So we take monocytes. We isolate them from volunteer blood freshly on the spot, use them right away in our models. We look at monocytes co-cultured with either red cell supernatant or microparticles and their result in cytokine production.

And we do this, we similarly see increase in the inflammatory cytokine IL-1 beta in response to microparticles either of fresh or stored red cells. That's here in the light blue. But you'll notice that there was marked increase in IL-

beta response after co-culture with the fresh red cell supernatant there in the red.

This pattern was a little bit different but slightly similar when we looked at IL-8. Here, in terms of the microparticles, we really only saw a significant effect in microparticles derived from older red cell. And though we saw an increase in the fresh supernatant here, it did not quite meet statistical significance.

We looked at TNFF. In the monocytes they're exposed to, extracellular vesicles alone or the supernatant alone, really no difference from controls, and in terms of IL-10, again in response to the supernatant from fresh red cells, saw a little bit of an increase in IL-10 production suggesting perhaps a bit of a mixed phenotype.

Now, we're not just interested in the response to the microvesicles themselves; we're interested in what happens to the monocyte's ability to respond to a new challenge? I'm an ICU physician, and so my patients are exposed to new

inflammatory stimuli and to potential infection all the time. And so we know that in the ICU, innate immune suppression, in addition to inflammation, but innate immune suppression is associated with adverse outcomes. And one of the ways that we can measure this in our patients is we can take blood from them, stimulate it ex vivo with LPS, and measure the amount of TNF alpha that it's capable of producing.

And when we look at this measurement across multiple diagnoses, we see that patients who have a persistently low ability to produce TNFF in response to LPS tend to do poorly. So here on the left is a figure from a study of children with multiple organ dysfunction. Those in the red boxes were those who did not survive and had persistently low TNFF or production capacity. The figures on the right are from children admitted to the ICU after trauma who, those in the gray boxes, went on to develop nosocomial infection. And you see they also had persistent innate immune suppression as measured

by decreased ability of their blood to produce TNF alpha in response to LPS.

Now, it's interesting. When we've started looking at transfusion in this context, we are seeing a pattern emerge in a number of different patient cohorts now whereby those patients who happened to receive older red cells have this persistent innate immune suppression, again that's measured by decreased TNF alpha production capacity.

So the figure on the left is from that same cohort of trauma patients. These are 29 of those patients who were transfused within the first 72 hours. And you can see that those who happened to receive older red cells had a persistent decrease in their innate immune function. The figure on the right is from 52 children with severe sepsis or septic shock, showing again a similar pattern. The gray-shaded area represents normal, healthy controls.

So back to the microparticles, we wanted to replicate this in our models. And so we took a

look at monocytes that were treated with red cell supernatant or microparticles and then stimulated with LPS.

And here we saw something a little bit different than the microparticles or the supernatants alone in that when we look at the fresh red cell supernatant or microparticles, we really didn't see much different in LPS-induced cytokine production of monocytes, but if we looked at the older red cell products, we saw that both the raw supernatant and the supernatant left behind when we isolated microvesicles, so the microvesicle-depleted supernatant, both caused a decrease in TNF alpha production capacity in response to LPS that was of a magnitude that was relatively similar to what we had seen in our patients.

To demonstrate that the monocytes are still alive and capable of producing cytokines, we also looked at the anti-inflammatory cytokine IL-10 and really didn't see a difference across groups.

So in thinking about mechanisms of this response, this immunosuppressive response that we saw with the older red cell supernatant, we knew that from our prior work that we saw this response at the miRNA level in our in vitro models, and so we've turned our attention to microRNA as a potential mediator of this response. MicroRNA, of course, are small, non-coding RNAs that post-transcriptionally regulate specific gene expression. And what's interesting is that red cells, while they don't contain a nucleus, do contain microRNA, and our work and work of others suggests that microRNA may change over time during storage.

The other thing that's very interesting about cell- free microRNA, one of the mysteries of their existence is how can you have RNA species floating around when you have RNases all over the place in your plasma? And so there are two ways that that probably happens. One is microRNA that are encapsulated within microvesicles, which protect them from the RNases. The other are

microRNases that are complex to chaperone proteins that similarly protect them from RNases. And so we took advantage of this property for the next series of experiments to get at whether microRNA might possibly be responsible for the effects that we were seeing.

And so we went back to our in vitro monocyte models, and when we added red cell supernatant from -- these were 30- day-old red cell products -- we saw the same suppression that we had seen before. If we treat with RNase alone as a control, we still see suppression. If we treat with heat and activation in order to inactivate protein mediators alone, we are starting to lose the effect but certainly some effect is still there. However, if we treat with heat to degrade protein followed by RNase, the idea being to degrade the chaperone protein followed by degrading any of the protein- bound RNA, the effect essentially goes away.

As a proof of principle, we looked at one of the microRNA that was most highly expressed

in the red cell supernatants and saw that indeed our treatment did result in decrease in that microRNA.

So ongoing work in our laboratory is working to understand differences in microRNA across storage in red cell products and what mechanisms they may play in red cell-induced immunosuppression in vitro.

So as a quick summary, and sort of dovetailing into the next talk and really following theme of certainly today and really the last two days, I think, you know, certainly given the complex nature of the blood products that we're talking about and the multiple soluble mediators therein, it's likely and not surprising that effects are probably mixed. And you probably have a mix of mediators within your product that have different effects. And it follows then, we've heard this story before, too, that when you're talking about microparticle mediated toxicity or effects of any other soluble mediators, that they are likely contact specific.

And so certain patients are more likely probably at risk for inflammatory toxicity versus immunosuppressive toxicity for example. And so it's really about balance. What is the balance of mediators in the product and what is the balance of susceptibilities in the patient.

So questions that remain -- there are many -- these are just a few. What are relative effects of red cell product-derived microparticles and other potential mediators of red cell toxicity, including what is the dose response relationship? Is it a linear relationship? Is there a threshold? You know, are there thresholds that we can identify that confer a greatest risk?

In terms of microparticles, understanding the toxicity is related to individual cells of origin and in general, whether total quantity is more important or is it more important where those microparticles are coming from? That certainly would have implications for how we might try to reduce that microparticle load.

And then again, of course, what are those patient- specific factors that would interact with microparticle- related toxicity or other soluble mediator effects? And can we identify certain patients who are at great risk of these toxicities? And lastly, can alternate red cell storage and processing methods prevent accumulation of microparticles or other soluble mediators in ways that improve clinical outcomes? I think that's the end goal.

And with that, thank you.

(Applause)

DR. BEUHLER: Thank you very much. Next, we have Dr. Phil Norris. Phil is the co-director of BSRI and professor of Laboratory Medicine and Medicine at UCSF. And I guess he'll take on the second part of this talk.

DR. NORRIS: Thank you. Okay, thanks for having me here. I know it's been a long session, and I think the FDA wanted clarity about what's going on in the red cell bag. But I think this is not just a pretty picture.

It's really -- I don't know, does the laser work? The big thing, of course, sorry. Okay.

So this fog I think is symptomatic. It's not just pretty. I mean, this is where -- partly I have jetlag so I'm down in the fog, but really, I think that as we're looking at this issue of TRIM and red cell quality, we're all sort of like the blind people looking at the elephant. It's difficult to tell what we have.

And so I'm going to give you one more little picture. I come from the angle of immune modulation. I do think that TRIM is difficult to define, and I'm going to go through some of the evidence in the literature. Jen and I just wrote a review article with several others in the room about this, and it's not clear as to whether TRIM exists or not, but we'll go through and look at that. And then I'm going to present some data that's not been published yet that we've been looking at some of the clinical trials that are going on.

So I came at this with a very open mind.

I don't know if TRIM exists or not. So we looked, and if we look at preclinical studies, these are in vitro studies, and they're also mouse studies primarily. So there are anti-inflammatory things that have been seen. So T regulatory cells are important in damping down the immune response, and it's been described that if you give a mouse a transfusion, the T regs can go up. Some of the T cell secreted cytokines can go down, and NK cell tested in vitro can go down after exposure to red cell units. TNF alpha, TGF beta -- TNF alpha can go down, TGF beta up. And then if you give aged or non leukoreduced red cell units to mice, they can promote tumor growth compared to fresh cells.

Now, there are balancing studies where it's been shown that neutrophil priming can be increased by red cell products, and both supernatants and the red cells themselves, and you can have increased pro-inflammatory cytokines, so really diametrically opposed to what we see on this side. And if we look at this, one way to do it is to count how many papers support each

viewpoint. And if you look at this, we have eight that are really more anti-inflammatory-focused. Their findings were. Three that found mixed effects or no effects of transfusion and four that said, well, they're really pro-inflammatory. So I'd say the literature is pretty unclear in the preclinical studies.

So if we move forward and we look at studies that are done in humans, I think these are important. If we think back of immune modulation, there was some really nice work done by Eldad Hod and Steve Spitalnik looking in mice. If they gave aged red cells to mice, they saw increased iron, but they also saw really a pro=inflammatory cytokine response. They went back and did that in healthy humans and they saw the same iron effect but they saw no effect in cytokines. So I really think that people are where we have to go to figure out whether TRIM exists and it's important for people, the people we transfuse.

So in people, it's been described in studies looking at transfusions. They have

increased IL-10 or decreased pro-inflammatory-type cytokines, decreased NK cell function, decreased monocyte function that Jen just told us about. And there are some pro-inflammatory effects as well. People have found pro-inflammatory cytokines in some studies, particularly with aged red cells, increased CRP or BPI. This is a bacterial permeability-inducing protein secreted by neutrophils, and they've also seen increased white cells. And again, the literature is fairly balance. I mean, you really can't get much more balanced than five, four, and six. Four showed mixed or no effects; five was more anti-inflammatory; six was more pro-inflammatory.

The thing I will say about the human studies that have been done in contrast to the mouse studies -- this is the problem with human studies -- is that we don't have randomized trials of people who are randomized to get transfusion versus those who are randomized not to get transfusion. These are all observational studies.

So that's a real limitation of the human studies, and it's a limitation of some of the work that I'm going to present in the next few minutes here.

So moving forward to what I'm going to talk about, and these again, we just really are analyzing the data at this point, so these are very preliminary data. But we had the opportunity to look at a number of parameters in subjects in the ABLE and RECESS trials. And so the ABLE trial was a randomized control trial of people who got fresh versus standard-issue blood performed mostly in Canada, and they had pretty good separation of the groups -- six days versus 22- day-old blood on average. They didn't get huge amounts of volume. It was 4.3 units. Importantly, these are patients in the ICU. So these are pretty sick patients to start with. They had a fair bit of mortality in the trial. They expected that going in so their primary endpoint was mortality. Secondary endpoint was multi-organ dysfunction syndrome (MOD) score.

So RECESS, as a complementary trial, and

what they did is they looked at complex cardiothoracic surgery patients. They don't expect as much death in this trial so their primary endpoint was delta MODs, the score. But similarly, they had a low volume, a median of three units transfused per patient, and they had good separation. This was actually aged versus fresh blood, so they had slightly older blood in the aged arm. And as everybody knows in the audience, there was no clinical difference in these two groups. So these trials really didn't answer the question, is that very oldest unit bad for you? Because that's not what they tested. They said, is the fresher unit better for you? And it's not, apparently, in these two patient groups.

So we went to the study organizers, and Phil Spinella was really my partner in crime on this. All this work was done in collaboration with Phil and his group. And in the ABLE trial we were able to enroll 100 subjects to look at a variety of remune parameters, and they balanced

out pretty well. So 51 were in the standard arm, 49 in the fresh, and there was a balance between males and females, and the apache scores were pretty well matched.

The RECESS trial, we had a slightly different set of subjects that we enrolled. So we had the standard versus fresh arm subjects, but we also had, importantly, nontransfused subjects. Now, these people were not randomized to nontransfusion, but they turned out not to need a transfusion. We still got samples from them at day zero and two, and I'll talk about the time points in a second. We also had 50 healthy controls. They weren't quite as balanced between the groups but I think because they're smaller groups than we had in ABLE but still relatively well-balanced.

So we looked at a lot of things. In the ABLE trial, we were able to look at cellular immune function. And what do I mean by that? We looked at T regulatory cells. So these are just looking at the phenotypes of the cells and do they

function as T reg cells? Then we looked at the ability of CD8 T cells to secrete interferon gamma to a stimulus. And we also looked at the ability of CD4 cells to secrete IL-17. So the TH17 response is thought to be pro-inflammatory.

So we looked at a number of anti and pro- inflammatory cellular markers. We also looked at extracellular vesicles, and we looked at their content and their phenotype. So what levels were they? And then we looked at a number of different cell types of origin. And I'll say parenthetically that I'm not going to show you data, in vitro data. We've done some more reductionous work in the lab to look at if an extracellular vesicle comes from a neutrophil or a granulocyte, does it behave differently from an extracellular vesicle that comes from a platelet? And we're finding that, in fact, that's true. Those data are going to be presented soon, but I can say that the platelet- derived EVs appear to have a more anti-inflammatory activity than the ones that are derived from granulocytes. Red cell

EVs, we don't find any immune function in our in vitro assays of how they work with monocytes. But the platelets in the endothelial and the white cell-derived ones do appear to have pro-inflammatory activity.

We also looked at a broad array of cytokines in the subjects, and we looked at a whole bunch of coagulation parameters in collaboration with Mitch Cohen.

So in the RECESS subjects, we looked at all the same parameters. This one, we set this trial up, or the ancillary study up a little after we did the ABLE, so we were able to add on thromboelastography and then Alan Doctor's group looked at nitric oxide contents in flux.

So today I'm not going to present all of these data. We don't have that much time. I'm going to focus on the immune data.

So with that, we did we find? So the first thing we looked at was the cytokine flux in ABLE patients. So these patients, we had a sample that was pre-transfusion. So day zero. And then

we had samples at days 2, 6, and 28. So looking at this, you can see that the majority of the cytokines that we looked at in this plot here didn't show much variation. There were a couple that came down. So, IL-6 started at a high level and came down by day two and down a bit more by day six.

Now, I'll point out that these subjects were all transfused. And the other thing I'll point out is that I've combined the fresh and the aged arms here because we found no difference. Much like the clinical trials, we found virtually no difference in any of the parameters between the fresh and the aged transfused in our immune coagulation and other parameters. So I think that's almost reassuring because the clinical trial results, the clinical results were the same in both groups as well. So I've lumped these together, and these are all transfused subjects. So the question that we have here is this due to somebody who is in the ICU who is getting better over the next couple of days or is it due to

transfusion? We can't answer that with the ABLE data, but we can at least observe.

So the next thing that we observed was not much fluctuation in the cytokines that are important for T -cell function. So IL-2 and IL-7 are T cell growth factors. IL-12 determines the immune bias of the T cells. Are they TH1 cells, which is what it biases them towards? Barrier function, this is important in the gut. And again, we don't see much fluctuation in these cytokines.

Inflammatory markers. So these are other inflammatory markers. We do see that MPO and PAI-1. PAI-1 is actually a pro-coagulant factor. This is plasma as an activating inhibitor. So these come down over time. And again, we don't know if it was the transfusion or just these people getting better as they're in the ICU.

And then we looked at more cytokine. We have a very big panel of cytokines we looked at. And again, we're not seeing huge fluctuations.

IL-10 is one notable one that starts out high and comes down over two days. We also looked at a number of chemokines and in IL-8 here is a pro-inflammatory chemokine. It also comes down over time. So summarizing the cytokines that we saw in the ABLE study, the general trend is either not much change or starting at a high level and coming down over time.

The next thing we looked at were the T cell functions I described earlier. So what we see here is the ability of CD8 T cells to secrete interferon gamma when they're stimulated, and there's really not a log of change in that over time, maybe a slight increase from day zero to two. T regulatory cells stay stable over the course of the ICU admission, and the interferon -- I'm sorry, the IL-17 secretion by CD4 cells. It's at a fairly low level, which is expected, and it stays fairly stable over time. So if we're looking for TRIM, this is not where we're seeing it.

The next thing we looked at were the

extracellular vesicles, and we looked at extracellular vesicles derived from a number of cell types. So by flow cytometry, we can actually gate on these small little particles on the left corner. I didn't put any introductory slides about this, but we worked very hard to validate the ability to detect the cell of origin of each of these extracellular vesicles. It's difficult because the signal is low. This is a particle that's small and doesn't have a lot of fluorochrome molecules on it, so it takes a long time and a lot of validation work to be able to detect these reliably, but I think we've got it down in the lab now.

So what we find to start with is that the total EV counts do fall over the first six days post-admission to the ICU and post-transfusion, and annexin-5 bearing EVs are only about four percent. This is -- the annexin-5 particles are on the left-hand scale and total EVs are on the right. So about four percent of the EVs that we found were expressing

phosphatidylserine, but they also fall in concert with the total EV counts.

Now, this is where it gets a little more interesting, I think. The red cell-derived EVs -- we know these people are getting red cell transfusions -- fall precipitously at day two post-transfusion and then bounce back up. Is this real or is this due to the ICU? We don't know. And this is -- the second thing we looked at were platelet- derived EVs. Again, both platelets and then CD62 positives, so activated platelet markers fall over the first two days and then gradually recover during the course of the ICU. And these fluctuations are really more dramatic than what we saw with the cytokine fluctuations, and certainly more dramatic than what we saw with cellular immune markers.

The next thing we looked at were lymphocyte-derived microparticles. So CD15 is sort of a pan white cell marker. CD19 is on B cells. CD3 is on T cells. So again, we see this same pattern of a dip over the first two days and

extracellular vesicles derived from lymphocytes.

The next thing we looked at was monocyte and myeloid-derived EVs. These did not show as much fluctuation as we saw in the lymphocytes or the red cells and platelets. So to my knowledge, this is the first time anybody's been able to do this detailed tracking of extracellular vesicles derived from various cell types over time in transfused patients.

And then finally, we looked at a few things that are important to T cells. So CD28 is a co-stimulatory molecule. For T cells, CTLA-4 is a negative regulatory molecule. So we see a fluctuation in CD28. We don't see much fluctuation in CD62 ligand or CD11B, which is an adhesion molecule.

Okay. So again, what we're seeing here in the ABLE study is people who have been transfused but they're also in the ICU. A whole bunch of other stuff is going on. So I wanted to go back now to the Recess patients and try to answer, is the fluctuation that we see in any of

these due to transfusion or is it just the underlying illness?

So if you remember in the Recess patients, we had people who went in for cardiac surgery. They thought they would need a transfusion. It turns out they didn't get one. We were able to compare those with people who did get a transfusion. Now we just got the key to unlock the data from New England Research Institute who did the statistical analysis here, so the figures aren't pretty yet because I haven't been able to, you know, make them in the nice programs. But what we found was that the T cell-derived extracellular vesicles fell over time from day zero to day two in those who got a transfusion but were stable in those who did not get a transfusion. We also found that CD62 ligand with activation markers on white cells falls over time. This is L-selectin in those who were transfused but not in those who were not transfused. And this was statistically significant comparing the two groups.

So we only have day zero and day two because in retrospect, I wish we got more time points in those who were not transfused but we said, oh, the nontransfused people aren't going to be that interesting so we'll just get day zero and day two. So the only comparison we can do is for the first two days for these.

Now, we also see that like we saw in the ABLE patients, the T cell markers go down over time and the B cell markers go down over time, but they do it in both the transfused and the nontransfused group. So this is something that we see a fluctuation. We don't think this is due to transfusion. This is potentially due to transfusion.

Now, this does not at all prove that transfusion caused this, this association, because these subjects were not randomized to transfusion versus nontransfusion. These two subjects, two types of subjects were different at baselines. One needed a transfusion, one didn't. The total EVs go down over time after cardiac surgery and

it's irrespective of transfusion. So that's not caused by transfusion. If we think back to the ABLE, we saw this same pattern. It seems like that really wasn't due to the transfusion itself.

The red cell markers, CD235 and CD108 is 74 and 78, these markers really don't change over time. So if we think back to what we saw in the ABLE patients, there was that steep decrease over the first two days. It doesn't seem to be due to transfusion, and these are different types of patient populations. We have relatively healthy people going in and getting cardiac surgery versus people who are arriving in the ICU and then going through their ICU time course. So again, observational studies, it's tough to make a firm conclusion but it looks like if we're looking for transfusion-related immune modulation, the red cell EVs are not changing much after transfusion.

Now, this is interesting. CD41A and then CD62P P- select and found on platelets. They both go down over time in these cardiac surgeries, subjects who were transfused, but not the ones who

were non-transfused. And again, these changes were statistically significant between the two groups.

So I think what we've identified here are some markers that might be associated with TRIM, to really prove that we need to hook on to a randomized trial of people going -- so, for example, a transfusion trigger trial, to find out people who are actually randomized to get a transfusion. They're not who would be the same at baseline, and then we'll see if these changes persist.

And then cytokines. There was one cytokine that was significantly different, but most of them showed this pattern where there was no affect at transfusion. And I think this is telling because if we think back in the literature, what have we looked at? We looked at what we can in the past. If we look at all those papers in the past, and most of them are looking at cytokine functions or cytokine levels. And if there isn't much change in cytokines due to

transfusion then, we're not going to measure it.

So in conclusion, TRIM is really hard to get one's arms around to convincingly show that he exists. There are some pro- and anti-inflammatory cytokines that fall after transfusion in ABLE subjects, and whether or not that's due to the transfusion itself or the critical illness, I suspect that a lot of these, like IL-6 and IL-10 are due to critical illness. They've been described to be elevated in critical illness.

T reg level and T cell functions are largely unchanged after transfusion in the ABLE subjects. And I think interestingly, a number of these cell-derived extracellular vesicles fall in the early period faster transfusion in the ABLE subjects who are ICU patients.

Going back to the Recess subjects, we were actually able to compare people who were transfused versus not transfused. We do see some cytokine modulation after cardiac surgery. The Recess subjects recapitulate the post- transfusion drop in platelet and lymphocyte EVs, and it really

looks like the platelet ones are associated with transfusion in the Recess study. Whether or not it's causative will require immune monitoring of subjects in transfusion trials where people are randomized for transfusion or not.

So with that I'll close and give acknowledgements. There are a lot of people who did, I mean, this slide should have about a hundred more people on it. Whenever you do a clinical trial there's a ton of people who contribute, but I'd particularly like to recognize Phil, who was really the co-PI of all of this. So thank you.

(Applause)

DR. BUEHLER: So we'll continue on now with our discussion panel. If anyone -- I see a question.

DR. GOLDING: So I'm Basil Golding. I'm with the FDA. And listening to these talks, I' trying to think all along, you know, what have I learned that we can use in terms of making these products blood -- red blood cells safer as a

product. So I have a few questions to ask.

First of all, I note that there's -- and I'm sure everybody is aware of it -- there's a discrepancy between the clinical data with all new red cells and the animal data. With the animal data, I'm getting the message from Paul Buehler's data that I'm aware of and Dr. Rakesh's data that yes, there is an effect of all the red cells, and it's quite dramatic in terms of mortality. And Dr. Rakesh has shown that even supernatants can cause similar effects, something in the supernatants, and I don't think it's a big leap of faith to say that it's mostly free hemoglobin and that the free hemoglobin is breaking down to heme iron.

So my first question is we are approving red cells based on one percent hemolysis. Is one percent hemolysis too much? Should we look at the human iron in those products? Should we wash the red cells that are of a certain age? So that's the first question.

Now I've got to remember what the second

question is.

So the second question actually has come to me. The second question is, well, you know, with Dr. Buehler and Dr. Rakesh's work, the hemopexin does a great job in reversing the effects. Well, doesn't it mean from a mechanistic level that the main problem is the heme and nothing else? Not the iron? Not the NO? Because Dr. Rakesh is showing that you get almost 100 percent improvement if you just use the hemopexin. Maybe I misunderstood that data. There's a big reversal. So it's mainly heme. So why are we worried about iron and why are we worried about, you know, well, okay, let's say there is an effect in certain situations -- in infections, in septic shock there's an effect of iron. The hemopexin reverses the effect of the heme. Have any experiments been done to reverse the effect of the iron? I would think of using iron chelators. With the ML, you could use nitrates. Should we be thinking in terms of public health, that there are ways to mitigate the effect if we start to see

adverse effects in a patient?

And also, this is an extrapolation that you, the panel, doesn't need to answer if they don't want to, but free hemoglobin has been used as a product, as a substitute for red cells. Hemoglobin-based oxygen therapies are based on that and we've seen a lot of toxicities. Well, maybe the panel is willing to say something about what could you do to mitigate those toxicities while using something like hemopexin or iron chelators or ICU experiments where they've used nitrates. And didn't see any beneficial effect, but maybe you have different answers to this. So those are the questions.

DR. BUEHLER: Okay then. It's a lot. Who wants to deal with this? I mean, I think one thing that, I mean, the effect of iron is quite clear, so maybe, Dr. Natanson, can you -- can you comment on any ways that you could mitigate the effects of free iron or non-transferrin bound iron in a situation, or even monitor a patient who is getting into trouble with

iron levels?

DR. NATANSON: There were an enormous number of questions that were asked and I'll approach the ones that I can remember. First of all, washing. In the canine model, we washed red blood cells. We took fresh blood and we took 42-day-old blood, washed it, and then gave it to the animals. And there was an interaction. The fresh blood when you washed it produced a worse outcome than the unwashed fresh blood. The old blood, 42-day-old when you washed it, produced a better outcome. So what happened? Well, when you wash, you lose a lot of red cells, and it's those old red cells that are going to produce all that cell-free hemoglobin.

DR. BEUHLER: Talk from -- talk from the podium.

DR. NATASAN: So washing old cells is good because the processing gets rid of the older cells that are going to hemolyze likely when you transfuse. Washing fresh blood is bad because you're going to disrupt a lot of cells that are

going to be less viable. So in terms of the statement "should we wash," fresh blood did the best. So I don't think washing is a viable alternative unless all you have is old blood.

If I can remember the questions. I agree with the statement that one percent hemolysis is probably not the best measure of what's in the bag. I guess two things come out of the bag or come out of the cells that are relatively important and that's cell-free hemoglobin and iron levels because I don't know how those two can go up unless you have a loss of viability of red cells.

So in terms of this meeting, and I've talked with Harvey a little bit about this, and Harvey, please, if I say something off base, you have to understand I'm not a blood bank or a transfusion person. And most of the people in this room forgot more than I'll ever know about this topic.

But for somebody who is not in your field, to me what makes the most sense is you've

got a bag, and the important thing about that bag is viability. And getting standard curves for cell-free hemoglobin and iron as two measures seems like a pretty good idea. But that doesn't tell you what happens after you transfuse. And if you take our model or get a pig model or any model, you can then take a look at what happens after you transfuse and you can again measure cell-free hemoglobin, iron, and see how well -- what your viability is.

And then the last thing I guess I would do is you have these solutions and these bags that have been used that we developed these standard curves, and then I would do randomized controlled trials in patients once I've established that in the bag it looks pretty good and in an animal it looks

(inaudible), and I randomize standard solutions that have been used in bags compared to other ones and measure these things in patients, randomizing the bags.

And that gets at the second one.

And then the issue if iron, which type of iron, whether it's heme or whether it's cell-free hemoglobin, or whether it's non-transferrin bound iron. I don't think any one of those is a good thing. I think the bacteria and the human species since time (inaudible) have been fighting each other, and they develop methods and techniques, cidofovirs and different ways of getting the iron, and then anything that has iron that you're releasing into the blood stream, which makes the bacteria's job easier, I don't think is a good thing. Whether iron has other toxicities that are directly related to which form it's in -- heme, cell-free hemoglobin, non- transferrin bound iron -- it may be important. It may be important in certain situations.

That's all the more the questions I can remember. Thanks.

DR. BEUHLER: Dr. Doctor, do you have --

DR. DOCTOR: Thanks. This was really a wonderful session.

I have, I think it's a fairly basic question about microparticle pharmacokinetics dynamics and clearance. It seems fairly clear that they have the potential to be impactful in many ways. It's not entirely clear, to me at least, how long they circulate the dynamics of the response to whatever sort of system they impact and in what tissue or cell is affecting the clearance. And if the clearance is in the MPS system as it might be, perhaps the tissue that is most affected is not accessible in the blood and need to look at tissue macrophages or lymph tissue to see where the most salient immune impact is. So I'm wondering what's already known about this, what should be done to sort of clarify it. And lastly, to allude to something that Chuck just mentioned, it seems even further complicated because post-transfusion, the elaboration and genesis of microparticles is continuing both in the blood that was transfused perhaps in the circulating native cells as well. So it seems like a very complicated pharmacology question that

might inform the questions that we're trying to answer.

DR. MUSZYNSKI: Yeah, I think that's an interesting question. Certainly, your data would suggest that the circulating monocytes themselves are capable of taking up the extracellular vesicles. Is it that 24 hours is too late to then look for evidence of microvesicles from the blood bags because they've already been scavenged or take up by the circulating immune cells, and in so doing, producing an immunomodulatory effect? The challenge would be could you actually do kinetic studies in patients post-transfusion at shorter time intervals? I think that's what you would have to do. I think it would be challenging logistically to do that, but that would be one approach.

DR. NORRIS: Yeah, so I'll expand on that a little bit. They have done some mouse model work where you can actually take the mouse and give it fluorescent microparticles and then cut up the mice at various times afterwards. And

within hours the microparticles are cleared from the circulation. They tend to land in the reticular endothelial system, spleen, but they also find them in places like the kidney that have high blood flow where they're flowing through, and they find them distributed throughout organs. So I think that your point is good that they probably are sequestered.

And then getting to your point, one of the things we're planning to do now in collaboration with Children's Hospital Oakland is to enroll people in a small pilot study to look at them after transfusion. People that are on chronic transfusion for sickle cell disease, we can then capture them with several time points over hours to try to get at that issue of the dynamics in the individual very early because I do think they go away within hours.

DR. ALAYASH: Can I have one quick note here? Recent paper in blood by a French group, they were basically saying that you lose heme and hemoglobin to the vascular endothelial system. So

in other words, in that short circulation time you start spitting out ucaga, which is damaged hemoglobin in addition to heme before you go in the rest of the vascular bed.

DR. BUEHLER: Anyone else? Dr. Weisel, do you have some insight from a coagulation perspective?

DR. WEISEL: Most microparticles are highly pro- coagulant as you heard, and generally in the circulation in healthy people, most of the microparticles are from platelets. But under other conditions you can get more microparticles from red cells or endothelial cells and other cells. But even in healthy individuals there's quite a load of microparticles from platelets, and they affect the clot structure, they affect thrombin generation, so they're important. And they increase greatly under especially prothrombotic conditions.

DR. BUEHLER: Okay. Dr. Klein, I think you were next.

DR. KLEIN: Yes, I think the question

for Dr. Weisel is, I found your studies very compelling and I'm a great believer in the coagulation function of red cells, but I wanted to ask you the other side of that, and that is in a long forgotten paper in the 1970s, I reviewed polycythemic patients at Johns Hopkins and found not only did they have increased thrombosis but increased bleeding as well, which I attributed to the abnormal platelets that those patients have. But in 1980, Dr. Robert Winslow and Carlos Monge and I did some studies in Peru at the highest laboratory in the world of patients with high-altitude polycythemia, patients with hemoglobin in the 20 range, and their cells are normal. And anecdotally, we were looking at oxygen delivery and not at hemostasis, but anecdotally, when we collaborated with some of the surgeons in the local hospitals, we found that they bled their patients prior to surgery. We asked them why, and they said if they don't, they'll bleed like all get out. So I'm wondering whether you looked in your system at normal cells

at very high concentrations, at hemoglobin of 16 to 20 and whether there's still a hemostatic effect or is there anti- coagulant effect?

DR. WEISEL: That's a really good question, and I wasn't aware of the high altitude studies. That's very interesting. We haven't done that. We haven't looked at very high hematocrit.

DR. KLEIN: Again, I guess it's maybe not relevant to this meeting because except for a few surgeons, no one transfuses patients to a hemoglobin of 16 to 20.

DR. WEISEL: Right.

DR. KLEIN: But I think it is an interesting question. Thank you.

DR. MUSZYNSKI: That said, there was a recent report looking at red cell-derived microparticles and the phosphatidylserine on the outside acting not only as a nitus for initiation of coagulation cascade but of your protein C, protein S mechanisms to break down clots. So that may be a potential mechanism at play.

DR. BUEHLER: Dr. Busch?

DR. BUSCH: Two simple questions and then one more point, discussion, and I'll do them one at a time, like vaso, so we can answer them.

The first one for Jennifer, the issue of microRNAs or possible even long, nontranslated RNAs which are now realized to be so important, within red cells or microparticles, do you think they're actually functioning within those cells or do you think they're being trafficked to end organs and mediating some regulatory function?

DR. MUSZYNSKI: That's a great question. Right now we have two hypotheses that we're thinking about. One is that they're taken into the cell the way that microRNA generally do in terms of gene silencing. But the other, and there are some very interesting reports coming out of microRNA acting as dams through the TLR pathway. And I think that that's probably, given the effects that we're seeing, the hypothesis that's going to win out in the end, but we're still trying to investigate that.

DR. BUSCH: So within the red cells or secondarily?

DR. MUSZYNSKI: Secondarily. So the microRNA, what we think was happening is that they were moving from inside the cellular space into the supernatant where then they're accessible to other cell types. And so then when they interact with immune cells, probably via signal transduction mechanisms through the TLR pathway.

DR. BUSCH: And then the second, for Rakesh, the role of hemopexin. Is this -- is this the hemopexin that's in the transfused unit or is this recipient hemopexin in terms of the real functional, potential dampening effect?

DR. PATEL: So the data I showed you was adding exogenous hemopexin, but it also raises a question, I guess, in response to the previous question. One of the things that I don't think we have a good handle on is if you buy hemoglobin and heme and iron, these are the key mediators of a gain of toxic function if you like. You also have to balance that out with what is the recipient's

status off the endogenous and protective mechanisms? The haptoglobin, hemopexin, the iron-binding proteins and so forth. And more importantly, I think, how do they change their function in time? So I don't -- to me it's, especially when we know that after transfusion, all these things are changing with time. So, you know, we design our experiments in a reductionist manner. We add something at the beginning at the onset of transfusion or given time but in reality, it's when is -- if you have some heme that's formed at X time, is that the key time for that heme to cause an injury? And if so, it matters what the hemopexin status is at that time. So it's a, you know, complicated, hand-waving answer to your question and the one earlier, and that's why I think it's not just one. I don't think that just one approach, picking one and going after it is going to be sufficient. In our experiments it was adding exogenous in those animal experiments.

DR. BUSCH: In both your data and Dr. Natanson's on the washing, et cetera, I think we

all appreciate that the hemolysis in the bag is, especially then to storage, is just the tip of the iceberg of what's going to happen when you infuse that product. And there's an enormous number of fragile cells that when infused will degrade and do all the damage. We also know that if we wash the cells in vitro, we destroy those and remove those cells. And is there a way to differentiate within the end of storage bag which are those fragile cells. And a lot the in vitro assays that we did under Mark Gladwin's guidance, we actually washed the cells and then we subject -- the cells that tolerate the washing are subjected to osmotic and oxidative stress. But what we're missing are the cells that were lost through the washing. And I'm just wondering, are there ways to bioemphamatically, if we could (inaudible) cells and then identify the (inaudible) population and capture them and characterize them versus the cells that were in the bag at the end of storage, or look at cells prior and subsequent to the washing or manipulation. I think in analyses that

we did for RBC-omics, we never really calculated what was the cell loss as attributable to the manipulations, and we figure out how to measure and quantify those end-of-storage fragile cells in some way that could be actually potentially converted into a relevant assay for the integrity of the cells through storage?

MR. PATEL: I think that's a great thought, and it's technically just challenging, especially when you think about I think Jason was mentioning yesterday, how you centrifuge a sample if your different product for those analysis.

So can we standardize that? I think that's a question we will have to ask ourselves, and I don't know what the answer to that is. Certainly, I think to me at least the protocols are in place to measure some of the key things that are relatively, you can measure theme a relatively straightforward manner whether it be the actual levels or after induction exposed to some stress levels of X, Y, Z, and you can get some pretty good insights from that.

But getting at your question specifically, I think that's going to be very challenging given the, just the heterogeneity of approaches and how much that impacts upon the result you might get.

DR. BUEHLER: Can I just add to that. We did a study, we didn't publish it, but we looked at a protocol gradient of stored cells, or prior to storage, and picked out different ages of cells as they were collected in a collection bag.

So after doing that, looking at the protocol gradient and then looking at the deformability of each age of cell, you could see differences.

What you possibly could do is take that approach at the beginning of storage, store those cells, and then look at what happens to those over time.

DR. PATEL: Can I just comment on that? And the challenge with that is that if you take the stored cells and do that protocol gradient, you just get a, start getting a smear.

DR. BUEHLER: We didn't.

DR. PATEL: You didn't? Okay.

DR. BUEHLER: We actually found a population that had decreased deformability. It was a very small population, and I would attribute it probably to the age of the cell in circulation, but I think it's possible to do.

MR. DUNHAM: I am Andy Dunham from New Health Sciences in Cambridge. I have a comment I'd like to make. First off, I'd like to say thanks to everybody for providing such compelling and incisive insights on that appears to me to be the state of the science.

The challenge I'm having as a person who's trying to make decisions based on all these insights, and I'm guessing the folks from the FDA, and caregivers who try to make decisions are really challenged by the breadth and diversity of the opportunities to improve the therapies for patients, and what I'd propose is a modest proposal on how we could frame these discussions.

And what I'm going to propose is that it

seems to me that the red cell bag is composed of three component. One of theme is a set of cells that survive and probably deliver oxygen to varying degrees. One's a set of cells or debris that are unrecovered and provide a burden a patient base and how the vulnerable the patient is, and the quality and the doses of red cells.

And the third component is crystalloids-based supernatant which contains varying amounts of plasma, iron, oxidation products, metabolites, blah, blah, blah, you know, so on and so forth, which we have varying degrees of understand of how they affect the patient.

The framework I propose is that we think about those three components of the product, and we price each of those components and think about the safety and the efficacy of each of those three components.

And then I think the fourth aspect of this framework would be to look for interactions and variability across the three components.

So I think that's what I've come away

with from these discussions. I think it excellent. Progress is being made. Great questions are being asked. The elephant is still in the bag, and it's hard to know what to grab onto to really affect, I think, and to help patients and for us to develop products, and for regulations, I think, to make those decisions.

So just a comment proposal. Thanks.

DR. BUEHLER: Anyone else have questions? Dr. McDonald.

DR. McDONALD: This gets to Dr. Nathanson's model which I was actually fascinated by, and it brought me back to the old days of hemoglobin based oxygen carriers.

I know there are a number of people in the audience who remember those good old days, and it seems to me that there are, and somebody would correct me if I'm wrong, that there was at least one infectiously lifalidy (inaudible) small animal lifalidy model that looked at hemoglobin and it was a bacterial strain specific effect on the lifalidy,

And there was one subclass of bacteria where it was really lethal in that model. I don't know whether it was a murine (inaudible) or it was a rat model.

But I'm wondering if different strains for lung infection that you would actually get the same result. That's one question.

And a sort of comment. With the small amounts of what is essentially uncrossling hemoglobin that could be released as cells go through and they lice through the system, I'm kind of surprised that they don't get filtered through the glomerulus unless the bacteria in lungs are eating theme up. Comment?

DR. NATHANSON: Your first question is would different strains of bacteria produce different effects?

DR. McDONALD: Uh-huh.

DR. NATHANSON: There's two things I would say is that viruses, every bacteria, all those infectious pathogens require iron. And so the effects may be modified because

there's probably some staff that are better to have, sidefiras that can take the iron off of transferrin, but I think they all, if you give theme bacteria, they'll all be very happy, I think, and it will not be a good even for the host.

Just an aside, this is an antibiotic treated model. So the animals have negative blood cultures, and they get -- and this was somewhat, I'm a clinician. I actually study septic shock and I treat septic shock patients. That's sort of my day job.

And what I was surprised at is that those iron levels going dramatically down, because they're on huge doses of antibiotics, and their blood is sterile, yet they're getting worse and iron is going somewhere. I presume it's very subjective it's going to the bacteria.

So then antibiotics in a deep-seated infection aren't doing what you want it to do, and most people with pneumonia take about five days to get better, and I now see why. At least this data

would give me one explanation.

And I apologize. Your second question? Can you repeat it? I went too long.

DR. McDONALD: Oh, I was surprised that if the hemoglobin is actually coming out of the, you know, as the red cell, all the red cells are licing, this is theoretically, and you're dumping basically what is uncrossling hemoglobin, which can dinarize and form 32 kildoltin dinarize and form 32 kiladoltin dimers that can go through the glomerulus, why isn't it being filtered by the kidneys, and if not, is there a direct interaction?

DR. NATHANSON: It is being filtered by the kidneys. One thing is that at least dogs, what we're using in canines is equivalent to a 12 to a 14-year old patient, and these animals are not affected. Their kidney function does not change. They don't have any damage from this level of kidney, which is, I guess, in terms of transfusion reactions, renal dysfunction is not a common abnormality.

But if I can turn the question over to you, Dr. Buehler, who have a guinea pig model where the hemoglobin does cause abnormalities.

DR. BUEHLER: Yes. So our model with hemolysis we do see acute kidney injury, and you can pick that up as I talked about yesterday through biomarkers like ANGAL, as well as higher transfusion volumes. There's no infection in this case, but at higher transfusion volumes, you can start to see renal tubal necrosis.

And we published that and last month there's a paper in mice done by Warren Zaypaul's group that showed basically the same thing, and that it can be blocked with, the effect can be blocked with haptoglobin.

The types of concentrations of hemoglobin are quite high. I mean, in Dr. Nathanson's model, you're looking at I calculate about 12 milligram per milliliter CMACs. That's high. And if you saw that in a cardiopulmonary bypass, that would predispose kidney injury right away.

But also in Zaypaul's paper in mice that developed AKI, there were very high levels of hemoglobin that were found in his studies.

DR. McDONALD: Well, one other thing that was occurring to me as hemoglobin-based oxygen carriers or efforts to produce them and obtain FDA approval are on the rise. And wouldn't this be a marvelous model to actually look at some of those because they would not, they're all modified to an extent that they would not affect the kidneys, one. And two, as they circulate, as a lot of these hemoglobin circulate, they produce meth hemoglobin, and the meth heme is one of the things that exchanges, and I'm just wondering what effect -- this looks like a very good, would be a very good model for looking at that.

DR. NATHANSON: And I hope you realize that there are people in this room that are on very different sides of the HBOC question that --

DR. McDONALD: No, that's okay. I mean, what we're looking for here is knowledge, really.

DR. NATHANSON: Let me first say that I

think that its an important area investigation, HBOCs, and I think that there's a possibility that our animal model could be helpful because I think the problem is that, there are many problems, but as I look at the experience with HBOCs and the animal data that I've done is, is that we didn't spend enough time determining how these things are metabolized, these different products.

And my concern is, is that sometime in that process of giving these HBOCs, you're suddenly have a large amount of iron, and potentially NO scavanging, or it could be any other theory. I don't pretend to know why HBOCs are toxic, but if they cause myocardial infarctions, if they did which I think I published some data which suggests strongly they did, nitric oxide scavanging would be a problem.

And I've seen a lot of the data from different products through unclear reasons why I had all that data presented to me, but there was an increased incidence diffusely of pancreatitis, which I assume is, is that the ampullae works on

nitric oxide, and if you scavenged it that's how you got pancreatitis and you got gall bladder.

It also was diffusely, and this is looking at Northfield's data, biopurist, hemolinks data, all of which I saw all of it. Not all. Well, most of it. And there is an increased incidence of septic shock and of pneumonia. And so I think depending on the host, if you dump a whole bunch of sulfur hemoglobin or at some point it's metabolized back to sulfur hemoglobin with NO scavanging, and there's a whole bunch of iron there's a problem.

So I do believe that I would be anxious to help in this field if anyone wanted to work with me, because I think we can look over days and times and see what happens with metabolism, and when these things come up, and then look at these things in models of infection, models of coronary disease, so I would be very anxious to help, because I think it's an important area of research, and if it could be accomplished that you could have a cell free hemoglobin that had minimal

toxicity, it would be a great clinical boon.

MR. McDONALD: Thank you.

DR. NATHANSON: But somebody else was on the other side of this, I'm happy to, if I misrepresented this issue.

MR. BUEHLER: Alayash.

DR. ALAYASH: I just want to shift the subject back to the red cell losing. And, in fact, if I can address Dove' question or suggestions, and the gentleman over here, red cells lesions versus HBOC, I this field is emerging. There's several hypotheses we've heard today. It's so really not very mature signs. So I think I will suggest we wait until these pathways are clarified, verified in the different parts,

(inaudible) before we really start adding ankylators or snow in the bag, or in hemopexin or (inaudible). All I'm saying is that there are still lots of areas to be investigated before we

really, we do not want to repeat the HBOC mistake where halfway through the (inaudible) of interventions, and therapies, and that didn't work, did it?

So what I'm saying, and this is the purpose, really, to end on this note, of this workshop, is to hear the science, see where we are, and I think we've learned a lot in the last couple of days, but it too much work to start adding things to the bag before we really know what's the culprit exactly.

DR. BUEHLER: Okay. Thanks. I really want to thank all the presenters. I think they did an excellent job, and it was quite informative. Thank you all.

And I guess we can have a 20 minute break now, yeah? Twenty? Twenty minutes.

(Applause)

(Break at 11:32 a.m)

DR. VOSTAL: And so we've decided that the best way approach for us would be to ask some

of the best minds in the business to actually summarize what we've heard in the last couple of days.

So that's what this session is going to be about. We've asked the discussants here to summarize their sessions.

So the first summary is going to be by Dr. Phil Spinella.

DR. SPINELLA: Well, thank you, Jaro. And, again, also thanks as well to you and the rest of the FDA for organizing this meeting. And clearly, you all think it's super important, and, hopefully, there'll be a way forward from here to effect some change.

So I was tasked to review both the initial session yesterday, which was determining the suitability of red cells for transfusion, and I'm going to also add the animal model for shock and trauma in my summary session here.

So, again, just to bring this back, you know, what was the goal of this conference? In general, it was to inform the FDA CIBA on

potential alternative criteria to license red cells storage solutions since the current approach doesn't account for direct measures of efficacy or toxicity.

So to be able to do this, we need to clearly understand transfused red cell physiology. We need to explore candidate measures of red cell efficacy and toxicity. And to do that we need to develop we think panels of measures that include metabolomics and systems biology principles.

We then need to test red cell quality measures and animal models for validation. Starting with small animal models and progressing to large animal models that we heard about yesterday.

We also need to develop appropriate reso transfusion indications for clinical trial methodology. Alan got to this a lot yesterday, and it's really essential.

I think a lot of the reasons why the trials we're performing now are not showing any differences is because we're transfusing red cells

when we shouldn't be. And if there's no potential for benefit, I think it makes it harder to see a difference between the interventions.

And then ultimately, we need to perform trials to assess if reso quality metric panels are adequate surrogates for clinical outcomes. Again, this could be done in trials in large animals or humans, and then as Jim mentioned yesterday, there may be different reso quality panels for different disease states.

Now, this may seem daunting and perhaps impossible due to the complexity and interactions between donor variability. The collection process, collection processing storage conditions as well as patient conditions.

Hey, now, let's be honest with ourselves. This will never be perfect. We're never going to come up with a way to license red cells that will completely account for each of these things. But we must, we have to improve from where we are right now regarding red cell licensing criteria.

And, you know, a little better is better than doing nothing at all. So this is basically my call to action to the group here to really work together as a group with the FDA, with the NIH, to make at the very least incremental improvements.

So, Alan yesterday gave us a talk on the influence of transfused red cell physiology, and his key points, I think, were that red cells, you know, clearly are a key note in the regulation of O2 delivery, or they help control vaso regulation.

We've all been taught in medical school that red cells were boxcars, right, they help deliver oxygen. But they also control the rail switch to help direct flow to the areas where they need to be.

He also spent a lot of time talking about transfusion decision-making, and, therefore, the ability to study transfusion efficacy. To be able to do this, we need to understand anemia tolerance, illness projectory, sequencing of transfusion with other interventions that support O2 delivery, as well as the ability to monitor O2

delivery as well as the dynamic reflexes that comprise hemostasis.

Another, I think, real important key point in his talk that we have to come away with is that according to red cell mass (inaudible) delivery there are many metrics here on the S axis he showed yesterday, but if the patient has a much higher hemoglobin, there's really only a risk of toxicity when you transfuse patients. And if patients are very anemic there's predominantly a chance of benefit.

But I think what we've been doing is been studying patients in this zone here where there's potentially no effect for response based upon the transfusion. So we have to understand this paradigm, and this may be why RIPI recess enable may have shown no difference, whereas the total trial that's (inaudible) group did from children from Ghana, they did show differences.

The children had a hemoglobin of 3.5 in general with an initial admission lactate of 8. And that group did show that in the older red cell

group, had a medical age of 33. It didn't go above 35, but the mean age of 33 those children did have reduced lactate.

So Alan's paradigm here yesterday may explain, or partly explain, some of the results that we're seeing. And it's very important for us as we move forward to take this into account.

In addition, he also highlighted that it's flow is more important than the hemoglobin content itself when it comes to oxygen delivery.

So this to me just highlights that we have to get away from using hemoglobin trigger trials to measure efficacy. That might be important -- hemoglobin trigger trials might still be important to look for safety, but for efficacy, as you can see, the hemoglobin level itself doesn't really reflect O2 delivery, and that's native hemoglobin.

If you transfuse the patient and increase that hemoglobin, the efficacy of that transfused red cell may also not relate to oxygen delivery.

The next talk was by Jim Zimring. He gave us insights into red cell quality, essentially of analysis and the take home points. I got from Jim's talk with that survivor and recovery and (inaudible) measures are suboptimal methods for measuring efficacy and safety of red cell units. Survivor and recovery itself clearly does not reflect actual efficacy. Just because a red cell is circulating throughout the vascular doesn't mean that it's actually able to improve oxygen delivery and consumption when needed.

And the hemolysis level itself is a partial safety analysis. This morning we even heard more about that. It's even less probably relevant as an accurate measure of iron related toxicity.

And then also Jim as well focused on good storers and poor storers, and how trying to predict who is a good storer or poor storer may also be misguided. I think this is an important point to draw out from yesterday's conversation too.

Just because a person is a good storer, and there is a higher amount of recovery and survival, that doesn't mean for sure that those red cells are also more efficacious. Just because they're circulating doesn't mean that they're an effective red cell.

And you could almost look to the platelet storage as another situation or that paradigm where with plain storage, we're learning now, or remembering from the sixties and seventies, that just because a platelet is circulating when it's stored at 22 degree Celsius, doesn't mean that it's very effective at being a hemostatic agent.

It's very clear that cold storage platelets is more hemostatic reactive, but it circulates less. So we can't relate circulation time, basically, with efficacy. That's my points. Or we don't know if true for red cells, and we shouldn't presume it.

Jim also made a point of stating that different patient disease states may require a

different panel of red cell quality metrics. I think that's clearly likely to be true. Whether that be patients with hemorrhagic shock, septic shock, hyproliferative anemia, or chronic anemia with hemoglobinnopathies.

So this is important. Something that we have to keep our eye as we move forward as a field.

Jaro talked to us about the three different ways that red cell storage solutions can be licensed. His main takeaway points were that the current process is designed to evaluate red cell products that are similar to conventional red cell products, and he stated that we need to expand the process to better evaluate novel red cell products. He emphasized that in vitro studies are not predictive of clinical performance, and we clearly need better pre-clinical tests that correlate with clinical outcomes. I think we all can definitely agree with that.

He also stated in vivo studies are

focused on red cell kinetics and circulation, but not oxygen delivery, again, emphasizing that we need pre-clinical and clinical methods to evaluate oxygen delivery whether it be in vitro tests or validated animal models.

So really emphasizing or reiterating the points that have been made in previous talks, Dr. Hess, Don, presented data on the clinical use of red cells for transfusion. Here for me the take home messages were as we all know, red cell transfusion is decreasing over time basically due to blood management principles.

I love the phrase that he used that we're moving from a just in case transfusion approach to a just in time transfusion approach which is, I think, true.

He also though explained to us that while we're decreasing red cell utilization now, in the near future, it may need to go back up due to demographic changes in the population.

Fifty percent of red cells are used for patients above years of age. This patient

population is expected to

double by 2025 so we're going to have to take this into account moving forward.

He also then reminded us that the recent and the past, I'd say almost now ten years of MPCs of (inaudible) platelets for patients with hemorrhagic shock has reduced red cell transfusion for severe bleeding, and also reminded us that now red cells plasma and even whole blood is being used in the pre-hospital setting to address hemorrhagic shock, and this being done in a few large trauma centers around the country, as well as actually around the world.

Dr. Klein gave us a presentation on the evaluation of red cell products with transfusion. The key points here was actually, again, donor viability and survival, and, I'm sorry, the donor viability in survival and recovery of red cells. He also showed us interesting data that red cell processing also causes variability in survival and recovery of red cells.

There was that great graph that he

showed that indicated survival recovery was different for irradiated red cells such as frozen verus standard, but we only license based upon the standard red cells.

He emphasized that red cells licensing criteria should provide a reasonable level of assurance or red cell efficacy and safety, and that kind of gets to the point that I tried to make up front. We need to get better, but we're probably never going to be perfect at this.

He emphasized that current evaluation criteria though somewhat arbitrary has in general service wide, but the changes that we make should be evidence-based. I think we can all clearly agree with that.

The biomarkers that are pursued should reflect red cell function, as well as clinical outcomes. These assays should be reproducible, and the methods for which the assays are performed should also be standardized.

This was highlighted more in Tracey's talk, but I think Harvey agreed with this as well.

Ultimately, the ideal evaluation criteria and the appropriate physical treatment are neither identified (inaudible)) or they would have been adopted long time ago.

So while that's true, that shouldn't paralyze us from moving forward, I know he didn't mean it in that way, but we shouldn't be paralyzed by the complexity of the problem ahead of us.

Jason then gave us a talk on predictive clinical value of in vitro measures and red cell quality. Here his main points were that red cell units should not be treated as if they're all the same. All red cell units are not created equal, just like plasma units are and platelet units aren't either.

He showed us very interesting data on multiple types of quality matrics. They're very different according to donor characteristics, collection of processing methods, storage conditions, and recipient characteristics, and whether they are biochemical, biophysical, or unit characteristics, the amount of variation here is

significant, so we have to take this into account when we start to examine red cell quality and metrics.

Moving on to the animal session, Mike Dubick from the U.S. Army Institute of Surgical Research, presented to us his swine models that he uses for shock and trauma. And here the take home messages were that swine models are the primary models that they use and most of the country actually uses for trauma and hemorrhage studies. The hemodynamic similarity in porcine models are very similar to humans, which makes it, which is a huge advantage of these models. The pig models are a little bit more hyper coagulable to human models, or human patients, so we have to be aware of that when we do these types of studies. It is encouraging that these models are very reproducible between centers that helps develop good multi center studies.

And actually after the talk, he didn't say this out loud to the audience, but he mentioned to me that transgemic swine that are in

development and he thinks soon to be available for a study may allow for human red cell transfusion too which will be a big help for us as we want to try to develop in vivo models of red cell efficacy.

Then Sylvain Cardin also from the USAI (inaudible) and from working for the Navy, presented to us his rhesus macaque models. And I thought this talk was very exciting for future use for our needs here. He showed us that humans are, that macaque models are the closest to humans except for the ape species. He showed us that there's extreme high protein hemology to include coagulation proteins between macaques and humans. He showed us that there was xenocompatibility between human products and these macaque models, and there's a lot more experiences coming out of the Navy to support this, and that the human dynamics hemostasis and immune response are also very similar to humans.

But, of course, as always limitations. He made it very clear, as you might expect,

there's a very high regulatory scrutiny when it comes to these types of models. There's a largely logistic burden on the animal lab staff when you perform these types of nonhuman primate models, and the cost is very high. He basically said that the out-the-door type price for an entire study would be close to 25,000 per primate.

So I was charged to identify (inaudible) sessions that I moderated the gaps and future directions. So I want to start with low-hanging fruit. And I think we need to start with prohibiting cherrypicking and licensing studies.

Jim brought this out yesterday. Someone else asked the question to the panel. And I think while it's difficult for industry to admit this publicly, I think we all have been told that at least that it happens. And if it doesn't happen, then prohibiting it from occurring shouldn't be a problem.

But we have to stop. I think it would be simple for the FDA to say if you've participated in a previous licensing, in a

previous survival and recovery study, you're excluded from participating in another one. It's that simple. And that would eliminate this issue that some are concerned about and keep on talking about.

But, again, moving forward from there, I think we as a community need to continue to work together with the FDA and the NIH to identify the most likely candidate surrogate measures for efficacy and toxicity.

Once we reach that point, then start to perform small animal models, perform small animal studies to determine which panel red cell quality metrics are associated with oxygen delivery, and toxicity.

The next step would be to test this panel of red cell quality metrics in large animal models. It could be dog models, it could be pig, it could be the nonhuman primate models, to see if there's an improvement in ultra delivery, if they produce toxicity, and if there's overall improved outcomes.

And, again, getting back to Jim's point, we might someday get to the point where we're doing this in trauma hemorrhage models, sepsis models, so on and so on, because it is probably likely that the quality metrics in these different disease states could be different.

And these surrogate markers that correlate highly with outcomes could then be considered by the FDA for licensing criteria, but that's a long way ahead, but we have to start now, and we have to, I think, work towards this ultimate goal.

Then ultimately, it's possible that animal model studies could be used to require licensing of red cell and storage solutions. At the very least, you could see in different disease states in these animal model at the very least improved O2 delivery.

Right now, all we're doing is assessing, basically, a hemolysis, and if they circulate, and even if circulation is three-quarters of the red cells are circulating, a quarter of them are not

based upon current criteria.

Additional gaps, and there was no way you can get me up here and not talk about whole blood, so this is my chance to stick this in there.

We have in this context of red cell licensing think about whole blood as a product. Whole blood use is increasing across the states as well as the world for patients with hemorrhagic shock, and there are new methods for processing and storing whole blood that will be coming to the FDA relatively soon. And I think we need to think about will those products be held to just the red cell licensing criteria? Will the whole blood that's stored at 4 degrees Celsius for out to 21, 28, 35 days? Will the platelets function in the whole blood? How will it be assessed? We need to think about platelet function in the context of whole blood too not just circulation time.

And to just emphasize why this is important, because in the past this hasn't been an issue because whole blood has basically not been

available. Not been available for a few reasons, but two of them have been the thought process that it can't be used past two to seven days at 4 degrees Celsius because the platelets do not function right, and also the standard of only using ABO specific whole blood.

The ABB is now permitting centers to use Group O. Whole blood is a universal donor. This is a big barrier that's improving whole blood availability. And then the concept of cold stored platelets is clearly being revisited, and there are now many centers throughout the country using whole blood stored at 4 degrees Celsius out to 14 to 21 days, and based upon some very intricate studies done mainly by the U.S. Army showing in vitro that whole blood or platelet units stored at 4 degrees Celsius have superior hemostatic function compared to platelets at 22 degrees for just five days.

So again, the clinical programs for whole blood are exploding, no pun intended, whether it be Pittsburgh, Mayo. The entire

Kentucky air ambulance system is bringing whole blood pre-hospital, and even the (inaudible) New Jersey, the university there the trauma program is starting to use whole blood. San Antonio and Houston, it's coming there as well too.

So if that wasn't controversial enough, I want to end on a, I think, probably a more controversial note. Oh, it's a question. Should previously licensed products be required to meet new criterial if and when new criteria are ever established?

I think we have to ask ourselves is it appropriate to hold a new product to one standard when the products that are clinically used have been held to a different standard.

I'll leave that as a question, and that's all I have. Thank you.

(Applause)

DR. VOSTAL: Okay, thank you, Phil, for that very comprehensive summary. So our next speaker is Dr. Thomas Raife, and he chaired the session on Methods for Detection of Red Cell

Processing and Storage Lesion.

DR. RAIFE: Thank you, Jaro. I'm going to keep this at a high level and brief, I think. So the Session III that my colleagues and I hosted really was very much focused on the modern approaches listed here in the OMICS realm, and it include all of these different elements and systems biology, and the way I want to sort of illustrate how I see it fitting into the program today and the industry is this sort of bubble chart approach where when we think of the IDC knowledge base, and primarily I'm talking about data, just volumes of data and phenomenology that I see our area as exploding in terms of the quantity of data that's coming out of these studies. And that by contrast, this area of quality efficacy and toxicity that we've been hearing a lot about today and in other studies by virtue of being much more complex, by virtue of being in vivo studies, animal studies, is less well developed, and the key thing I think is the relationship between the area of OMICS studies and

these studies is that the OMICS studies really need the quality efficacy toxcity type studies in order to inform it.

As Dr. D'Allessandro mentioned to me yesterday, we had this capacity to characterize red blood cells orthogonally and so many different ways now, you tell us what you want or understand about it in terms of efficacy, toxicity, and quality, and we can help figure that out.

The last piece that I think is very important just to keep in mind clinical practice, which the knowledge of clinical practice really lags behind the science of transfusion medicine quite a bit.

So in terms of -- I'll talk about challenges and opportunities with the OMICS. I think that from what I hear and talking to my colleague from a technical standpoint, I don't see that there are huge challenges. I'm not hearing a clamoring for new instrumentation that can analyze molecules in another way.

I think, rather, that the technology has

driven a huge outpouring of data from the folks working with that right now, so I don't think technical challenge is a huge -- perhaps the one thing that one might comment in here is the potential for false discovery with the volumes of data that are coming out.

So in terms of challenges here, one of the big things, and we talked about this at the dias yesterday, is the analytical problem, and that is the problem of big data. So just in our very small pilot study of twins, we have approximately 70,000 data points coming out of that study, so I can imagine that the red study with 14,000 participants in GWAS alone would be I don't know how many orders of magnitude larger than that.

So there are enormous databases sitting out there that I think by and large are sort of bricked, as Dr. Paulson would have said, and that there's enormous advantage to be gained if those can be consolidated into a database that would be much more comprehensive than what we have right

now, but that exercise being a challenge in and of itself is, I think, tiny compared to the problem of analyzing big data, which is a much more monumental task than I think I can appreciate, and, perhaps, many people as well.

As Dr. Paulson pointed out, when you look at analyses of the expense and time and energy it take to really do comprehensive big data analysis, it begins to usurp the expense of actually generating the data itself.

But I think that's a huge opportunity that the field is poised to potentially take advantage of. So right now, I think that our group feels that the big challenge is translation. We've generated a lot of data. We have made great inroads in the understanding of phenomenology of red blood cell biology and biochemistry. The question is what's important. And so the answering the question of what makes red cells work, what makes their quality, et cetera.

I think that the immediate possibilities for translation (inaudible) opportunities are in

the more conventional measures of efficacy, quality, and toxicity, things like in vivo recovery, hemolysis, that sort of thing.

The much bigger challenge is with the more experimental assessments of quality and efficacy, things that we heard today, and I call theme experimental because there are numerous ways of approaching all the various problems, and there is yet to emerge a convention in terms of what constitutes quality and toxicity.

And then, ultimately, the big challenge is the capacity that the area that I'm working in in OMICS has for really sort of translating into precision medicine and transfusion medicine, so clinical indication specific properties or red blood cells as was alluded to earlier today.

So that will be a future goal. And then not forgetting the challenge of clinical practice which from my point of view the, transfusion remains a rather blunt tool for therapeutics for clinicians. Just visiting with our hemonc docs earlier this week about their workflow in the

hemonc clinic for cancer patients, you know, the example was, well, we get a 70-year-old woman who comes in. She's chronically transfusion dependent from her chemotherapy. She's seen not not by the hemonc doc, but, rather, by a nurse practitioner, who is running on protocols. The patient complains that she's tired. They look at her hemoglobin, whether it's actually above or below the threshold they use for transfusing, they order two units of blood.

And so that's about as much data as goes into the decision to transfuse. Not only whether to transfuse, but how much to transfuse.

So it remains blunt and old school, and far from precision medicine. So however much energy we put into improving the quality of red blood cells and making them boutique for various indications, we have a huge, huge challenge out there with clinicians in terms of getting them to understand those nuances.

Opportunities on the side for OMICS, near term opportunities, I think we are poised to

make progress on some of the more conventional issues related to quality. For example, in the red study predicated on the notion that they can measure hemolysis in about 14,000 blood samples and do and do GWAS, they'd be positioned to make some, bring a bunch of progress in terms of understanding the genetic determinants of hemolysis which could very easily lead to an improvement in that particular parameter of stored blood.

Also, in in vivo recovery, the work that we're doing and the work that Dr. D'Allesandro is doing I think are very much poised to making improvements in terms of the emergency metabolism, the understanding of energy metabolism in red blood cells which gives rise to the opportunity to identify donors by phenotype and/or modulate the storage milieu to improve that. Things like anaerobic storage being possibilities.

In the sort of intermediate term, I mentioned that consolidation of databases of the OMICS data, and then big data analysis. It's a

huge opportunity to vastly expand the systems biology knowledge of red blood cells.

Sample repository sharing. The database has a sample repository as a huge resource, and there are others out there as well. This is database consolidation, integration, curation and really sort of big data analysis that, as Dr. Paulson pointed out not only would vastly impact and improve our understanding of red blood cell physiology, but also extrapolates potentially to human physiology. And as I learned last night, by number red blood cells are 80 percent of the human cells in the human body, so they're representative of a large amount of human tissue.

Other opportunities. So the long-term stretch goals I mentioned sort of precision RBCs or boutique RBCs ways of accomplishing that through the work being done in the OMICS field would be selecting donors for their genotype or phenotype that stores better in one way or another, or has other medicinal properties that are better for one kind of scenario, or patient,

or another.

Also, the way that would inform ways to modify blood cells in the way that, for example, Dr. Hess' AS7 modified the storage milieu for red blood cells by adding sodium bicarbonate, and altering the ph.

And then thirdly, manufacturing. And here what I'm getting at is that I think we have to be mindful of the fact that somewhere along in the foreseeable future, these cells likely will be coming out of bioreactors, and in order to understand how to do that properly, some choices are going to have to be made, some selections about what kind of phenotype or genotypes are going into those bioreactors in order to optimize the properties coming out.

Lastly then, I'm sort of looking at the entire milieu of these various activities, and I see kind of four camps here. Again, the efficacy, quality, toxicity camp that is working very hard and is working with vary challenging animal models and human models, and also taking advantage of new

technology to gain insight into how the efficacy of red blood cells can be measured.

Then there is the biology biochemistry, OMICS camp, which as I said is really very much beholden to the first camp in terms of trying to figure out where to target their understanding and their efforts.

And then there's the regulatory camp, which is looking for these first two camps in order to inform it as to what kinds of things they should be looking for.

And then there's the manufacturing camp, which is basically we'll take marching orders from the regulatory camp in terms of what it needs to do in order to produce these for the consumer, which is not listed here, but is an important piece of it too.

And so I'll leave you with that framework.

(Applause)

DR. VOSTAL: Thank you, Tom. That was a great summary.

So our next speaker is Dr. Harvey Klein, and he chaired the session on Animal Models, Oxygen Delivery and Profusion.

DR. KLEIN: Thank you. I really appreciate being referred to as one of the best minds in the discipline these days. I consider myself one of the oldest minds in the discipline, but maybe not more than that.

And just a passing thought on the red cells to come out of the bioreactor. If they're all of the same life, and they die at the same time, what a load of iron that might give to you. But just a passing thought.

So I'm talking about the animal models in the first session, and it was quite interesting to me. Dr. Buehler described to us the various categories of animal models. For example, those that are designed for efficacy, those that are designed primarily for toxicology, the healthy models, and then the combined models which can evaluate not only efficacy and toxicology, but potential, the additive effects of various

therapies.

He described the model that he used, as we know, over the many years, the guinea pig model for transfusion, and its potential for demonstrating at least two candidate biomarkers of injury.

And parenthetically, I think that in general I've seen a lot more that's helpful for injury or toxicity of red cells than really for the efficacy of red cells. And I'm not talking about oxygen delivery, although that's part of what I'll be discussing, but efficacy, which is I think what the FDA wants, and they may not be identical.

But in any case, he showed how in tissue you can

> (inaudible) a HI1 Alpha in his model, and pimidimazole, which may turn to be useful models, or useful biomarkers for injury.

Now, in his model, one does see kidney injury. What you don't see with transfusion in

many of the other animal models this may be an issue or problem, but it also might be an advantage that if this animal model is more sensitive, perhaps that would pick up issues with toxicity in red cell products that perhaps other animal models would not.

The second presentation was by Dr. Tim McMahon, and Dr. McMahon described really elegant studies of human red cells transfused to nude mice, and in this model, anesthetized mice can be infused with the equivalent of two units in human being of packed red cells either fresh or stored, and by the use of dyes and other methods, you can look at their adherence, the adherence of the red cells in the pulmonary vascular bed.

You can also show when ATP and oxygen delivery, or oxygen saturation crash, and they seem to correlate with a number of things, but most importantly, perhaps, ICAM4 up regulation, so maybe this would be a potential biomarker again of toxicity showing when red cells adhere to vasculature pulmonary or otherwise.

He then told us and demonstrated that you really do need to then validate this in animal models, in a mouse, for example, with mouse red cells so that we know that this isn't something peculiar to the mixture of human cells in an animal, and he showed that with mouse red cells, the same things pertain.

And again, with very elegant studies, he showed intravascular microscopy and how it can be used to look at blood flow and hypoxia.

Still and all, in terms of efficacy, certainly, there's no real correlation yet with any kind of clinical outcome, and that's something that's absolutely necessary.

The third presentation was by Professor Marcos Intaglietta, who many of you know has spent his life looking at capillary issues in a very elegant model. But what he started with was a mathematically derived engineering model, and he confessed that these models usually fail when you put them into practice, but went ahead anyway and described it in great detail, and it's a model

that looks at the viscosity of anemia, which is understudied. The viscosity of polycythemeia is well known, but at the other end of the spectrum it hasn't been well studied, and how that changes with transfusion, and how that changes flow characteristics and, therefore, the delivery of oxygen. A very nice construction, and he modeled it with a half unit, a unit, two units, and additional units of red cells transfused.

And what he showed in his constructed model was it's very small changes in hematocrit can lead to very large changes in both flow, and, in fact, it took six units of red cells in that particular model to make the delivery of oxygen from hemoglobin as important or more important than the changes in flow characteristics.

So it appears at least in this model that it's hard for red cell oxygen delivery to trump flow. That flow is so incredibly important.

But then he took this construct and he validated it in his hamster model. And although he was very skeptical that these engineering

models would work in vivo, in fact, the hamster model seemed to confirm most of the predictions that he had regarding flow and delivery of oxygen, and one of the very impressive observations, I thought, was that, in fact, very high viscosity fluids given to anemic animals resulted in a 40 percent increase in delivery of oxygen.

What was even more interesting to me is that he found evidence of increase in inflammatory cytokines which may be an epiphenomena, but he thought might be in some way related to the delivery of oxygen, and, in fact, if he decreased the inflammatory response by giving his animals dexamethazone the effect of oxygen delivery disappeared.

Unfortunately, as he pointed out, this model does not distinguish between the effects of fresh red cells and very old red cells. It's only the consistency of the findings that are more variable with the older cells, but this couldn't be used at present to help us in anyway distinguish the quality of cells one from the

other in various preparations. But he felt that the model could, in fact, be tweaked in order to do so.

The final presentation I confess was the most interesting one for me, and maybe that's not, because I'm not an animal model person in general, but Professor Harold Schwartz from Dartmouth, a professor of radiology, who, again, confessed to us that this isn't his field of discipline, and yet seemed to ask some very pertinent questions to this particular field of discipline, described a variety of techniques for assessing tissue oxygenation potentially with transfusion, the techniques in general and particularly applicable to transfusion.

Unfortunately, many of the techniques either are no longer in use, or result in damage to the tissue so that they really can't be used on multiple occasions, or used continuously to help you evaluate what's happening in a animal model.

But to me, I was very impressed by his description of EPR oximetry, which he's been using

for several years, which employs India ink and other dyes to look at oxygen delivery in tissues, which can be applied to organs, internal organs as well as to tissues on the skin, which is where we generally have looked for oxygen delivery in the past.

And I confess that I asked Dr. Glynn perhaps a decade ago if heart, lung, and blood couldn't find some technologies to look at oxygen delivery during or before and after transfusion because we really don't know much about the correlation of oxygen delivery to different tissues or organs in the outcomes of patients.

It seemed to me that this particular technique might well be applicable to some of our animal models, and might be helpful not only in distinguishing the various preparations of red cells, which is what this meeting is all about, but also in telling us how oxygenation of different organs and tissues does, in fact, over a long period of time and multiple transfusions relate to outcome.

So I thought that all of these presentations were helpful in terms of trying to get at the issues that the FDA wants to get at, but none of them really at this point in time have provided really a path forward for helping us to distinguish the quality red cell from the not so good red cell, and I think that remains to be seen.

I also think in terms of trying to discern what a better red cells is, and in addressing one of Dr. Spinella's comments, I think what we have now it works. I think it's hard to say that it doesn't work. It may not work optimally, or at least in some situations it may not work optimally, and in other situations it may work, but it may have some toxicity as well.

Before we decide that it doesn't meet some new criteria, and that what we have is not good enough to be licensed, I think we really do need to make sure that our criteria are outcomes based, and that what's good is not necessarily nonusable because there may be something better.

So I would say that in answer to one of the questions you posed, Phil, when we have evidence, outcome evidence, that a newer preparation or a better preparation is available for red cells or whole blood, then in an economy like ours, the good usually drives out the bad.

Thank you.

(Applause)

DR. VOSTAL: Thank you. That was, as always, a very insightful conclusion.

Our next speaker is Dr. Paul Buehler. He did the last session on Potential Mechanisms of RBC Transfusion Associated Toxicity.

DR. BUEHLER: All right. So I'm not going to go as detailed as everyone else did because clearly you heard our discussion as well as our speakers' presentations today, and that should be pretty fresh, but I'll just briefly overview.

So the issues that we're talking about a lot here is issues that are associated with storage of red blood cells. But I think the

issues that we're also concerned with here are issues that are associated with not only storage of red blood cells, but extended storage RBCs, processed RBCs, stem cell derived and bioengineered RBCs.

So while a lot of this discussion has been about storage and what the effect of storage time is, these are also critical things that we have to look for in the future in terms of how we're going to regulate these and what scientific data makes sense in applying to regulatory processes, if any.

So, obviously, in the bag, the red blood cell undergoes changes over time, and these lead to hemoglobin accumulation whether it's in the bag, and we know that it's not just in the bag, it's post-transfusion as Dr. Nathanson pointed out and others have pointed out.

And so this is an issue that we not only have in terms of understanding what's going on in the bag, what's going on following transfusion of different levels of units of red blood cells.

Closely related to this issue then is the issue of nitric oxcide. Nitric oxide's depleted in red blood cells that are stored in the bag, but also nitric oxcide is depleted in the circulation primarily by hemoglobin. So there's close relation here that needs to be studied a bit further for us to really understand how we can translate to any human situation.

The other issue, of course, is that the breakdown product of hemoglobin is typically heme and this can be released in tissue, and it can also accumulate in a bag, so differentially, what are the effects from bag accumulated heme as well as post-transfusion released heme?

Then we have the issue of iron, which I believe the accumulation of iron is not so much a bag related issue, but what happens post-transfusion typically by erithrophygecitosis you get a large increase in iron, and this is clearly a problem in things that are associated with infectious disease, and clearly increase mortality associated with certain disease state

conditions, but we also need to learn now we can translate this to humans.

We also learned about coagulation thrombus formation, which is probably an underlooked area that is associated with transfusion, but it's an important one. But understanding a thrombus in an animal is very difficult to do, and identifying whether or not coagulation changes have led to a clot that has led to deaths, or to some other toxicity is also a challenge. So we need to work on understanding really what coagulation changes mean.

The issue that was brought up here as well as here and here are the issues of microparticle formation. And this is an area that I've gained a lot of new respect for over the last day or so, and I think microparticles form in the bag. I don't know if they form post-transfusion but they likely do, and understanding what essentially these do in terms of overall toxicologic effects is quite important.

The issue of MIRNA or micro RNAs is an

intriguing one and needs to be explored further to look at an understanding of what these components can do.

And then finally, immune modulators post-transfusion. Clearly transfusion related immune modulation is important, but we're not quite sure how important it is, and how the balance of immune modulation is tipped following transfusion.

So in all of these areas, I think we have clear gaps where we can provide a better understanding through kind of basic research to provide a scientific basis for any type of regulatory action in the future if that's necessary.

And the other thing that I would say is that when we're talking about toxicity or toxins, it's always important to know what levels are relevant here. Whether it's in the bag, whether it's post-transfusion, and what can be done if particular toxins can be identified as being really causative for any type of adverse event,

and how to intervene as a result of that.

And with that, I will end, and thank you.

(Applause)

DR. VOSTAL: Thank you, Paul. That was a great summary. So this bring us to the conclusion here, and we've asked Dr. Paul Ness to give us some final thoughts.

So Dr. Ness is the Director of the Division of Transfusion Medicine and a Professor of Pathology Medicine and Oncology at the Johns Hopkins University School of Medicine.

DR. NESS: Thank you, Jaro, and thank you all for your indulgence in staying on to this late hour, I know afterwards, and I understand that there are traffic jams to be fought, and planes to be caught, and trains to get to, so I will try to be somewhat brief.

I think I was given this assignment. I was a part of the Planning Committee, and, unfortunately, I missed a couple of the meetings. And, therefore, I think when I ended up looking at

the agenda, I saw all of a sudden I was going to be given this ominous task.

And before it gets too farther along, I should admit that even though I don't think these issues will come into too much play in what I will have to say, I have to disclose that I am a consultant to the New Health Sciences company that's trying to work on an anaerobic blood storage system, and I also work with Terumo BCT, who, among other things is trying to work on a pathogen and activation system for whole blood and red cells.

But the most challenges I think the talk that was given to me is something I always dreaded as I was a young turk sitting in the audience many years ago, because I always never really wanted to be the person who got up at the end of one of these things and doing what I'm doing and sort of summarizing what has happened and where we should go.

And maybe that's for fear that perhaps when I'm doing that nobody thinks that I have any

new data of my own or new thoughts of my own that I can give.

The other problem in terms of doing this is they wanted the slides in advance, so that I'm supposed to give final thoughts on this meeting even before I was here a day and a half to hear this wonderful potpourri of scientific, and medical, and sort of philosophic thought.

So that added to the challenge to some extent. But I will go forward assuming, and maybe adding a few thoughts that aren't on the slides based on what I think, you know, I predicted would happen, and what has developed subsequently.

So the first thing we've all, many of us all know this slide, or know these details, but, you know, we're faced with a big controversy that started really, we've known about it for a long time, but I think what really heightened this was the report by Kochetal from the Cleveland Clinic on Cardiac Surgery, the retrospective study that suggested that old blood had a two time increased mortality.

And we're all aware, and we've seen some of the data actually presented in different formats here that we now have a number of randomized clinical trials, an ARIPI study in neonates, the recess study in adult cardiac surgery patients, the ABLE study in critically ill ICU patients, and the TOTAL study in small children in Africa, who are critically anemic.

And most of these studies are giving us some reassurance that perhaps old blood isn't so bad, although we also have admitted if you look through the slides that the old blood in these studies really isn't that old, although to some credit here in the TOTAL study that was done, that blood was

days of age, and it did seem to correct the anemia without major potassium problem and corrected acidosis pretty effectively in those very, very sick children.

On the other hand, we're still faced with this dilemma, and we've seen pictures of these ugly red cells that go along as we go, and

we know that in storage of red cells, we have problems with impairing red cell deformability and all the other things we've talked about today.

And, actually, all of this stimulated a number of these tremendous presentations we saw here today. So we now know more and more about the storage of red cells, and, perhaps, the goal of trying to normalize the storage of red cells going forward, and it will be wonderful to see if we can ultimately apply some of these materials to this clinical problem we're facing.

But I don't think it's totally resolved, and this isn't data that was alluded to, from Johns Hopkins that we published recently in transfusion, and when we looked at our large data base of transfusions over the past six or seven years, we were able to carve out a relatively small population of patients, who for whatever reason got blood only stored greater than 35 days, and compare it to blood that was stored for up to 28 days, and even blood that was stored even for shorter periods of time.

Only about seven percent or so of our blood it happens probably is now being distributed greater than 35 days, but what you can see here as you look at the odds ratios in all patients who only got blood stored greater than 35 days, ICU patients in particular, or elderly patients in particular, we have a signal here that those patients who only were getting this 35 day and older blood may have some clinical compromisers, or even enhanced morbidity and mortality that we should be cognizant of.

So in terms of the question that I would ask in terms of a final thought, is the age of blood controversy resolved? Well, we've talked about the randomized clinical trials in several adult and pediatric patients populations which show no difference in flesh versus blood store for longer periods of time looking for adverse effects and function issues, we're all anticipating the results of a study called INFORM that will be presented at the AABB, which is a very large pragmatic study being done in Canada and other

parts of the world looking at almost, over 30,000 patients, and those will be presented there.

But in general, there's been some thought that all of this is reassuring, and we don't need to worry about this anymore.

On the other hand, I just showed you retrospective studies in patients receiving older blood, which I believe suggests some problems. We heard the elegant studies from the NIH of very old blood in very sick animals suggesting problems. And we also heard alluded to, and actually presented in some ways, the studies by Eldat Hard and Steve Spitalnic showing the problems with the iron load that comes from older blood, which may worsen outcomes.

So my conclusion, and I hope, perhaps, some of you will share this, is that shorter storage periods, or better preserved (inaudible) systems may still be prudent options, and, hopefully, there will be additional studies coming forth to confirm what we need to do, or we need to make some changes with the existing systems.

In terms of making changes, what is the first question I'd ask, and the first question I would sort of ask is do we really need blood storage for 42 days, or could the blood transfusion system in the U.S. handle shorter storage limits?

And I think the answer in the U.S. is probably we could handle this. As I mentioned, probably at our hospital, only about seven percent or so the blood is transfused beyond

days, and I believe from our regional Red Cross that supplies us, a small proportion is going out at that point as well.

I understand that the military and developing countries may have more significant problems and may not be willing to adjust the storage time, but I think that's something we ought to consider.

And I understand that when we present this question to community blood centers, they may say, well, if we do this, outdating may increase, and if outdating increases, cost may increase.

And I guess I would say, so what, because we need to understand that in blood components, platelets, for example, if we want to have adequate platelets on the shelf, and we're going to outdate them at five days, we're going to have to have some outdating. We have to understand that the problem of outdating is going to be a part of the overall cost of having a adequate supply of blood, particularly for surge periods when we didn't predict the need.

And so we, I guess, have to try to not be the collective wimps we've been, and try to argue with our administrators in hospital and healthcare to say, hey, if we really can make blood better by, for instance using blood cells that are shorter life, or platelets have adequate supply by understanding that we're going to outdate some of them because we can't totally predict the need, it's gonna cost more, and it shouldn't bother us to be supporting the fact that what would cost more probably would be better for our patients that we serve.

And I also believe that the problems could be mitigated by the adoption of newer technology and advance transport systems that are used in other industries. We as an industry often complain that, you know, we can't deal with split inventories. That's supposedly a big deal. Well, it shouldn't be.

And we also should understand that, for instance, a grocery store understands that to get relatively fresh produce out on the shelf, there's pre-delivery costs. Some of the groceries are going to be there for a certain period of time. They are going to outdate because nobody wants to buy rotten lettuce, and we're all paying for all of that process, so the same logic really should apply to blood, and other industries can handle it. Why not us?

Another thing that I thought looking at the program we weren't going to address, and I think we really didn't address it, but some us are optimistic and enthusiastic that someday we may have pathogen reduced red cells. We now have

pathogen reduced platelets, maybe we'll have red cell some day.

And we need to think about how are we going to assess their clinical promise, and whether they had problems for patients. We're going to have to repeat all of these clinical trials that we did for the current stored red cells to see if there's long-term toxicity for those things. So I think that's going to be an evolving issue going forward.

And I guess the final question that I sought of wanted to summarize with is with all of this information that we're gathering, will FDA be able to adopt guidelines for industry that will allow enhancements of blood storage solutions to be licensed and implements that would be cost sensitive.

I think we all heard John Hess tell what the trials and tribulation even with the existing systems were to get products licensed for AS7. And what he didn't say, but we also know in deference to sort of this cost argument that we're

making, is that even though it finally got licensed, not many people are using it because people think it costs more and doesn't have much clinical benefit.

I think that's all very unfortunate, and I think we have to really think and help the FDA to know that.

So how much of the new knowledge about red cell storage would we need to apply to applications to license modifications of blood cell solution, because if we create a situation whereby manufacturers are creating a system and are forced to do a whole series of tests that just are so expensive for minimal gains in transfusion efficacy, they may not do it, and for whatever reason, we may not be willing to pay for it.

So if the guidelines and requirements become too burdensome, the clinical advantage it's a better solution, so it might help patients may never be realized, and I think that would be very unfortunate, because I think we all understand that ways that we can improve the storage of red

cells that potentially can meet more than needs of patients, perhaps subgroups of patients, we need to continue to study that, and, hopefully, we will go forward in partnership with the FDA to come up with ways that some of the information that we've heard today, some of the information that manufacturers are working on potentially could be implemented and, actually, used in our systems.

So the final sort of slide is just to allude to this commentary that I was fortunate enough to work on with Simone Glynn and Harvey Klein, the red cell storage, and what we believe, and I would urge you look at the commentary if you haven't seen it, is we're sort of at the end of the beginning. We raised the question prompted by the Club Study in 2008, NIH and other manufacturers responded by doing a whole host of grant supported work some of which you've heard today, a number of clinical trials were proposed and completed to give us some reassuring answers, but I don't think we're there yet, and there's lot of work to do in our careers before we finish,

and, hopefully, in the long run, our patients will benefit from it.

So thank you for indulging me the final remarks.

(Applause)

DR. RAIFE: All right. Thank you very much for those parting thoughts. I only have a few minutes or a few seconds here to say thank you for everybody who participated in this workshop, especially the steering committee that planned out the agenda, and the sessions chairs that kept the speakers on focus, the speakers themselves for taking the time to come present their data, and share their knowledge.

And, also, finally, I'd like to share with you some of the names of the people who worked behind the scenes to make this happen, specifically, Dr. Monique Gelderman and Jennifer Scharpf, and the folks managed to travel, and finally the sponsors that made this workshop possible.

So thank you very much. This concludes

the workshop, and travel safely.

(Applause)

(Whereby, at 1:08 p.m. the PROCEEDINGS were adjourned)

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CERTIFICATE OF NOTARY PUBLIC

COMMONWEALTH OF VIRGINIA

I, Carleton J. Anderson, III, notary public in and for the Commonwealth of Virginia, do hereby certify that the forgoing PROCEEDING was duly recorded and thereafter reduced to print under my direction; that the witnesses were sworn to tell the truth under penalty of perjury; that said transcript is a true record of the testimony given by witnesses; that I am neither counsel for, related to, nor employed by any of the parties to the action in which this proceeding was called; and, furthermore, that I am not a relative or employee of any attorney or counsel employed by the parties hereto, nor financially or otherwise interested in the outcome of this action.

(Signature and Seal on File)

Notary Public, in and for the Commonwealth of Virginia

My Commission Expires: November 30, 2016

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