JANET LEE: Thank you so much for the invitation to present this talk. This is a 20-minute talk, and it's dedicated to the Department of Medicine house staff. And so the title is "Red Cell Transfusion, How Old is Too Old, From Biology to Controversy." And the three main objectives for the talk is one, I'll briefly introduce the concept of the red cell storage lesion and the surrounding controversy. And since there are numerous studies experimentally in preclinical models, I'm not focused on that, but really, in this brief talk for simplicity's sake, focus on human data in healthy volunteers on age red cell transfusion effects and how it disrupts iron handling and homeostasis and two, disruption in nitric oxide availability.

So as you all know, red cell transfusion is a common practice in medicine. It is one of the World Health Organization model list of essential medicines. It's unequivocal that red cell transfusion saves lives, but that's actually not the question here.

The question is, can we improve upon that? And so the controversy is surrounding the in-vivo consequences of red cell storage lesion. And the question persists, how old is too old?

So what is the red cell storage lesion? Well, red cells undergo senescence in cold storage. And there's a series of biochemical and biophysical membrane changes as well as metabolic changes that occur, all collectively termed the storage lesion. And they increase as the duration of storage increases.

However, the FDA allows for storage for up to 42 days in the United States. And the requirements are one, in-vitro, the red cell unit bags have to show, on average, less than 1% hemolysis at the end of storage and two, in-vivo the average 24-hour post-transfusion recovery is greater or equal to 75%. And that's utilizing radiolabeled autologous red cell units in healthy volunteers.

So the performance standard for red cell units has changed very little since its original description in the *Journal of Clinical Investigations* in 1947. And there they adopted an arbitrary value of 70% post-transfusion survival. Now, that's gotten modified just a little bit to 75%.

The other notable finding is that the longer the storage duration, the more non-viable the red cells there are and faster their removal rate. In fact, it's within the first couple of hours. And that may get more important later on, as I'll show you some more current data. And this is an original graph from that paper looking at 24-hour post-transfusion recovery as a function of the number of days the red cells are stored. And they're looking at a variety of different solutions.

So there's clearly limitations in the performance standard for red cell units. One, it's an arbitrary designation laid by expert opinion. And then number two, it does not address physiologic endpoints or clinical outcomes of transfused recipients.

So, how old is too old? Well what we're talking about is the limits of storage duration, or the very oldest blood in the sixth week of storage, or 35 to 42 days. And the evidence thus far in humans, healthy volunteers, is that physiologic endpoints have now been measured, and there is increase in extravascular hemolysis, as well as increase in intravascular hemolysis. And so let's just start off with a little bit of definition so that we're all on the same page. And so I can't talk about red cell transfusion without talking about iron, and iron is, as you know, an essential element, and it's particularly useful because it can accept and donate electrons. And so it's at the core of many proteins that power the energy house of the cell, catalyzes enzymatic reaction, and, of course, oxygen transport to all the cells in the body. And so if you have a deficiency in the iron, you have anemia, either in the delivery or uptake, and so you can have iron deficiency anemia or anemia of inflammation.

If there's defective synthesis of protoporphyrin, which is a heme backbone, then you have sideroblastic anemia. The heme molecule then gets incorporated into the globin subunits. And if there's a defective synthesis in globin, you have thalassemia. And all of this have to properly be synthesized into the hemoglobin tetramer.

So this is a review from a number of years ago. But I think it's particularly instructive to this day. And this is about normal iron distribution. So I'll talk about three points.

One is that 1 to 2 milligrams of iron enters and leaves the body each day. So it's absorbed through the gut, through our diet, and then there's sloughing of mucosal cells. But the majority of the iron is in hemoglobin. And what is under appreciated is that macrophages of the liver and spleen, previously known as the reticular endothelial macrophages, degrade senescent red cells and provide most of the usable iron in the body.

So here's another review from others, and there's an error in this diagram. But they're far better artists at *New England Journal* than I could be. So I wanted to use this to make three points about regulation of iron transport, because it becomes relevant when we talk about aged red cell transfusion effects. And so ferroportin is the iron gate by which iron moves out of cells. And so here in the gut lumen, you have iron from the diet taken up.

There's a reductase activity that occurs in the brush borders. And then it gets into, through DMT1, Divalent Metal Transporter 1, ferrous iron, and then it has to go through another little transporter, the iron gate, the ferroportin, on the basolateral surface. And this ferrous iron, actually gets oxidized to ferric iron by ferroxidase, which is on the transmembrane surface here called hephaestin.

So transferrin has a high affinity constant for ferric, Fe3-plus iron. So this actually should be Fe3-plus. And so this is a main mechanism of iron transport. And iron is transferred to developing red cells, as well as if an excess gets stored in the liver. And so the other component is hepcidin. And that's a peptide that gets synthesized by the liver and regulated by a variety of factors, and it's the master regulator of iron homeostasis. And this blocks iron efflux by actually binding to ferroportin, the iron gate, and allows for the internalization of ferroportin, degradation in the lysosomes. And so if you have a deficiency in hepcidin, you have iron overload, for example, hereditary hemochromatosis.

If you have excess, you have anemia, either iron refractory iron deficiency anemia or anemia of inflammation. And wouldn't it be great if we had a hepcidin antagonist to address the issues of anemia of inflammation or iron refractory iron deficiency anemia? So the age red transfusion effects, when you have age red cells and they get transfused into healthy volunteers, there are two points I'd like to make.

One is data has been shown, by actually Mike Risbano and Dr. Gladwin here in our division, that there is increased intravascular hemolysis. So the stored red cells, because of their fragile membranes, hemolyze, cell-free hemoglobin, so the tetramer, which has ferrous oxyhemoglobin, Fe2-plus, and so consumes nitric oxide in the vessel through the dioxination reaction. So ferrous oxyhemoglobin reacts with NO, and then you get methemoglobin and nitrate.

The other thing that I don't show in this diagram is that arginase gets released from the red cells, and it actually diverts away the substrate for nitric oxide synthase. And so you have reduced NO synthesis, NO scavenging. All of this contributes to endothelial dysfunction and presumably organ injury.

So there's another group that's shown increase extravascular hemolysis. And that occurs where the age red cell breakdown occurs in the mononuclear phagocyte system of the liver and spleen. So macrophages or macrophage wannabees actually take up the red cells and catabolize the heme. And then ultimately, ferrous iron gets made. And this gets transported out of ferroportin.

There's a ferroxidase activity that's GPI-linked called ceruloplasmin that oxidizes to ferric iron. And then, as you know, transferrin now binds, because of its high-affinity constant, to the ferric iron. But that capacity is overloaded when you give acute delivery of old red cells, and there's the creation of the non-transferrin-bound iron, which is basically ferric iron bound to albumin, citrate, et cetera. And an increase in percent transferrin saturations, this acute rise actually signals the liver to make more hepcidin, which then negatively regulates the ferroportin.

So this disruption in iron handling is of concern because many preclinical studies have shown that this potentially provides an alternative source of iron to pathogenic bacteria or possibly due to impaired phagocytic capacity. And so this is sort of the paradigm I'd like to show you with the data that's currently available, so extravascular hemolysis overwhelming the normal capacity of the mononuclear phagocyte system and disruption in iron handling and appearance of non-transferrin-bound iron. So Hod and colleagues at Columbia University showed that when you transfuse healthy volunteers with the very oldest blood, compared to fresh blood, these are autologous, you see an acute rise in total bilirubin and a small rise in conjugated bilirubin, so suggesting that most of this is indirect and consistent with extravascular hemolysis.

In contrast, markers of intravascular hemolysis, such as LDH and haptoglobin, were not increased. So here they also show a rise in serum iron here, [INAUDIBLE] And these are the individual healthy volunteers data. And then here also, the rise in percent transferrin saturation and the formation of non-transferrin-bound iron.

When you take sera from these volunteers that were transfused with the very oldest blood, you can see that it enhances the proliferation of bacterial pathogen in-vitro compared to those that received fresh red cells. So does this actually increase infectious risk in susceptible hosts? Actually, that study has not been performed in patients, and nor will it ever be.

But the concern is potentially there, particularly in ICUs, where we see a lot of susceptible hosts. And this is a particularly interesting study, a cross-sectional analysis of 75 countries containing over 1,200 ICUs over 14,000 patients. And on that day of the study, 51% of the patients in the ICUs were considered infected. 71% were receiving antibiotics. And longer the ICU stays, it's no surprise, had higher rates of infection and doubling of the ICU mortality, as well as doubling of the hospital mortality rate.

So bacteria have co-evolved as well. And they utilize metal chelators. And they're called siderophores, which have a very high-affinity constant for ferric iron. In fact, the affinity constant is much higher than mammalian proteins, such as transferrin and lactoferrin. So theoretically, they could actually scavenge iron from transferrin and lactoferrin.

But if that were not enough-- this is enterobactin, one of the classic siderophores-- endogenous catecholes, such as norepinephrine or epinephrine, are chemical mimics of siderophores. And they've been shown, at least in-vitro, they can complex wit transferrin and lactoferrin and pull the iron away and making that more available to bacteria. So this is something that I think is of intense interest to folks in the field.

So now let's switch over to intravascular hemolysis. And that's age red cell breakdown and free hemoglobin release within vessels, which, of course, is not a normal occurrence. This is a diagram from Dr. Risbano's paper that was recently published. And I kind of focus on the right side where there's red cell hemolysis, increased cell-free hemoglobin. And you see that NO is scavenged through the dioxination reaction, formation of methemoglobin and nitrate. This is an irreversible reaction. And then there's release of red cell arginase in addition, where actually it diverts away L-arginine from [INAUDIBLE], which then reduces the amount of NO being synthesized.

So reduction in NO synthesis, increase NO scavenging, all together produces less NO signaling, less guanylate cyclase signaling, and impaired relaxation of smooth muscles. So in their study, they used a model of forearm blood flow responses to acetylcholine. Now, acetylcholine, as we all know, is a neurotransmitter. But also, it can cause endothelial-dependent vaso dilation through the release of nitric oxide. And so as they increase the acetylcholine in these volunteers and transfused them with the very oldest blood compared to the freshest blood, you see there's significant impairment in the percent change from baseline, which was their primary outcome of interest.

This is associated with increases in cell-free hemoglobin at post-transfusion on day 42. And this is kind of a remarkable finding, because we haven't endogenous proteins that scavenge cell-free for hemoglobin because of its toxic effects. And so even one unit of red cells after to produce this finding, I think, is kind of remarkable and then increases and arginase 1 after fresh and very old blood transfusion.

So intravascular hemolysis in the forearm blood flow responses to acetylcholine model in healthy volunteers you see a reduction or endothelial dysfunction and release of cell-free hemoglobin and red cell arginase. Now, could this contribute to ischemia in microvascular beds and consequent organ injury? And that actually has not been shown directly.

So I end with donor red cells because Dr. Gladwin wanted me to talk about this, but the genetics of donor red cells and how it might impact storage. And so, as I discussed, host and pathogens have co-evolved for the battle for iron acquisition. And pathogens have influenced human genetics. And this is a story of mankind.

Over 100 red cell membrane mutations have arisen, thought due to selective pressure of malaria. And this is actually a beautiful world map of hemoglobin S allele frequency, which is the point mutation in the beta globin that causes sickle cell anemia. And this is the prevalence of allele frequency is heavily concentrated in malaria-endemic regions, sub-Saharan Africa, the Mediterranean, Southeast Asia. And this is the current malaria map.

This is from a review article by Rees and Gladwin and colleagues a number of years ago in*The Lancet.* And so donor red cell genetics may influence storage integrity as well as performance standard. And this was a paper that we published with Mark Gladwin, looking at this hemoglobin AS, or sickle cell trait, and looking at storage characteristics of hemoglobin AS compared to hemoglobin AA or the [INAUDIBLE] type. And you can see storage dependent increases in hemolysis, as well as, paradoxically, a blunting of osmotic hemolysis. And really, that is due to the fact that their membranes are more rigid.

This is actually where we come is the mouse model, looking at the hemoglobin AS mouse model, a hemizygous Berkeley mouse and similar storage properties. And we show accelerated clearance of these cells following storage in mice. So I leave with you how old is too old? Well, what I've shown you is that physiologic endpoints have been measured currently in healthy volunteers that now challenge the FDA criteria.

Clinical trials have not been designed actually to test the question of the very oldest blood. In fact, over 13 randomized controlled trials have looked at storage duration. And Dr. [INAUDIBLE] will go over the clinical data. But none of them have addressed this particular issue, mainly because it's deemed unethical to actively age blood to one arm of a cohort. And so that trial will probably never be performed. But the NIH, the UK, and the Netherlands, have actually now changed practices to limit storage duration to 35 days. And so the controversy continues. I thank you for your attention.