UNITED STATES FOOD AND DRUG ADMINISTRATION

RED BLOOD CELL WORKSHOP

PRE-CLINICAL EVALUATION OF RED BLOOD CELLS FOR TRANSFUSION

Bethesda, Maryland

Thursday, October 6, 2016

PARTICIPANTS:

SESSION 1 - Introduction - Red Blood Cells as Transfusion Products

Workshop Introduction:

PETER MARKS, MD, PhD
Director, Center for Biologics Evaluation and Research
Food and Drug Administration
Silver Spring, Maryland

Influence of Transfused RBC Physiology upon Recipient Oxygen Delivery Homeostasis:

ALAN DOCTOR, MD
Director, Pediatric Critical Care Medicine
St. Louis Children's Hospital
St. Louis, Missouri

Insights into RBC Quality, A Century of Analysis:

JAMES C. ZIMRING, MD, PhD
Chief Scientific Officer, Bloodworks Northwest
Director, Bloodworks Northwest Research Institute
Professor of Laboratory Medicine, Professor of Hematology
University of Washington School of Medicine
Seattle, Washington

Supporting a Strategic Research Agenda in Transfusion Medicine at NHLBI: RBC Products:

SIMONE GLYNN, MD, MSc, MPH
Branch Chief, Blood Epidemiology and Clinical Therapeutics Branch
Division of Blood Diseases and Resources
National Heart, Lung, and Blood Institute
National Institutes of Health
Bethesda, Maryland

PARTICIPANTS (CONT'D):

SESSION 2 - Determination of Suitability of Red Blood Cells for Transfusion

FDA's Approval Process for RBC Transfusion Products:

JAROSLAV G. VOSTAL, MD, PhD
Chief, Laboratory of Cellular Hematology
DHRR, CBER
Food and Drug Administration
Silver Spring, Maryland

Clinical Use of RBCs for Transfusion:

JOHN R. HESS, MD, MPH, FACP, FAAAS
Professor of Laboratory Medicine and Hematology
University of Washington, Harborview Medical Center
Seattle, Washington

Evaluation of RBC Products for Transfusion:

HARVEY G. KLEIN, MD
Chief, Department of Transfusion Medicine
NIH Clinical Center
Bethesda, Maryland

Predictive Clinical Value of in vitro Measures of RBC Quality:

JASON ACKER, MBA, PhD
Senior Development Scientist, Canadian Blood Services
Professor, University of Alberta
Edmonton, Alberta, Canada

Discussion Panel:

JASON ACKER

PARTICIPANTS (CONT'D):

ALAN DOCTOR

JOHN R. HESS

HARVEY G. KLEIN

JAROSLAV G. VOSTAL

JAMES C. ZIMRING

Panel Discussion Leader:

PHILIP C. SPINELLA, MD, FCCM
Associate Professor and Director,
Translational Research Program
Division of Critical Care, Department of Pediatrics
Washington University in St. Louis
St. Louis, Missouri

SESSION 3 - Methods for the Detection of Red Blood Cell Processing and Storage Lesions

Omics of RBC Storage Lesions (Proteomics, Metabolomics, microRNAs):

ANGELO D'ALESSANDRO, PhD
Metabolomics Core Director
University of Colorado Denver
Department of Biochemistry and Molecular Genetics
Anschutz Medical Campus, Aurora, Colorado

Systems Biology of RBC Storage Lesions:

BERNHARD PALSSON, PhD
Professor of Bioengineering, Professor of Pediatrics
Systems Biology Research Group, Department of Bioengineering
University of California San Diego
La Jolla, California

PARTICIPANTS (CONT'D):

Genetics of RBC Storage -- Studies of Twins:

THOMAS J. RAIFE, MD
Clinical Professor (CHS), Director of Transfusion Services
Department of Pathology and Laboratory Medicine
University of Wisconsin-Madison
Madison, Wisconsin

REDS-III RBC-Omics Study:

MICHAEL P. BUSCH, MD, PhD
Co-Director, Blood Systems Research Institute (BSRI)
Senior Vice President, Research and Scientific Affairs, Blood Systems, Inc., Scottsdale, Arizona
Professor of Laboratory Medicine
University of California
San Francisco, California

Discussion Panel:

MICHAEL P. BUSCH

ANGELO D'ALESSANDRO

BERNHARD PALSSON

Panel Discussion Leader:

THOMAS J. RAIFE

SESSION 4 - Animal Models of Oxygen Delivery to Tissues by Transfused Products: Oxygen Delivery and Perfusion:

Potential Biomarkers of RBC Function in Animal Studies:

PARTICIPANTS (CONT'D):

PAUL BUEHLER, PharmD, PhD
Pharmacologist, Senior Scientist, Laboratory of Biochemistry and Vascular Biology
DHRR, CBER
Food and Drug Administration
Silver Spring, Maryland

Correction of Anemia: Humanized and Other Mouse Models:

TIMOTHY J. McMAHON, MD, PhD
Associate Professor of Medicine
Duke University
Durham, North Carolina

Hamster Microcirculation:

MARCOS INTAGLIETTA, PhD
Professor, Bioengineering, Institute of Engineering in Medicine
UC San Diego, Jacobs School of Engineering
La Jolla, California

How to Measure Effective Oxygenation of Target Tissues:

HAROLD M. SWARTZ, MD, PhD, MSPH
Professor of Radiology, Department of Radiology
The Geisel School of Medicine at Dartmouth
Lebanon, New Hampshire

Panel Discussion:

PAUL BUEHLER

MARCUS INTAGLIETTA

TIMOTHY J. McMAHON

HAROLD M. SWARTZ

PARTICIPANTS (CONT'D):

Panel Discussion Leader:

HARVEY G. KLEIN

Shock/Trauma Resuscitation: Swine Models for Shock/Trauma Resuscitation Research:

MICHAEL DUBICK, PhD, FCCM, FACN
Supervisory Research Pharmacologist
Chief, Damage Control Resuscitation Program
U.S. Army Institute of Surgical Research
San Antonio, Texas

Non-Human Primate Transfusion Models:

SYLVAIN CARDIN, PhD
Chief Science Director
Naval Medical Research Unit-San Antonio
JBSA Ft. Sam Houston
San Antonio, Texas

Panel Discussion:

SYLVAIN CARDIN

MICHAEL DUBICK

Panel Discussion Leader:

PHILIP C. SPINELLA

Other Attendees:

PRADIP ALKOKAR

ABDU ALAYASH

ROBERT ALLISON

DAVID ASHER

HELEN AWATEFE

PARTICIPANTS (CONT'D):

JIN HYEN BAEK
DEBRA BECKER
LUCA BENATTI
CELSO BIANCO
SANDRA BIHARY-WALTZ
JERRY BILL
BARBARA BRANTIGAN
JOSE CANCELAS
SHARON CARAYIANNIS
ALLENE CARR-GREER
MAITREYI CHATTOPADHYAY
TIFFANY CHEN
LAUREN CLARK
PAMELA CLARK
RAFAEL CORDERO
WILLIAM CREWS
MICHELLE DABAY
NEETU DAHIYA
SILVIA DE PAOLI
MICHAEL DIOGUARDI
LARRY DUMONT

PARTICIPANTS (CONT'D):

ANDREW DUNHAM
ANNE EDER
JAY EPSTEIN
RICARDO ESPINOLA
SUE FINNERAN
JOEL FRIEDMAN
BASIL GOLDING
ALAN GRAY
MARYANN GRUDA
SALIM HADDAD
EMILY HERZOG
LOUISA HESCHEL
ELDAD HOD
MARY HOMER
P. ANN HOPPE
GREGGORY HOUSLER
ORIEJI ILLOH
SHREE KOUSHIK
SANDHYA KULKAMI
ANJU KURIAN
JIE LI

PARTICIPANTS (CONT'D):

YING LI

VICTOR MACDONALD

SHERRY MATHEWS

ELISABETH MAURER

FANTAO MENG

PHYLLIS MITCHELL

CAROL MOORE

NAIM MOSES

NINA MUFTI

WENDY PAUL

HEATHER PRATT

KENNETH REMY

MARIA RIOS

JOHN ROBACK

STEPHEN ROGERS

NEETA RUGG

NINA SALAMON

JENNIFER SCHARPF

JAN SIMAK

EMILIA SIPPERT

RUTH SYLVESTER

PARTICIPANTS (CONT'D):

JOHN THOMAS

DEDEENE THOMPSON-MONTGOMERY

AMY TSAI

SACHA ULJON

MANOJ VALIYAVEETTIL

NICOLE VERDUN

BERYL VOIGT

KERRI WACHTER

STEPHEN WAGNER

PATRICIA WEDDINGTON

LISABETH WELNIAK

FEI XU

AYLA YALAMANOGLU

SCOTT ZIETLOW

SHIMIAN ZOU

* * * * *

P R O C E E D I N G S

(8:00 a.m.)

DR. MARKS: We are going to go ahead and get started. Good morning. And thank you for attending this Public Workshop on the Preclinical Evaluation of Red Blood Cells for Transfusion. The workshop has been planned and co- sponsored by FDA in partnership with the National Heart, Lung, and Blood Institute; the National Institutes of Health; the Department of Defense; and the Office of the Assistant Secretary for Health of the Department of Health and Human Services. The workshop will include presentations and panel discussions by experts from academic institutions, industry, and government agencies.

Just to provide some context for the presentations that follow: advances in the transfusion of patients over the past decade that have included the implementation of lower transfusion triggers have reduced the clinical use of red blood cells by several million units per year in the United States, and this reduction in

use ultimately relates at least in part to concerns regarding safety and efficacy.

The motivation for holding this workshop derives from the recognition that currently available pre-clinical tools for assessing the quality of stored red blood cells fall short of an ability to reliably predict the safety and efficacy of transfused products. Likewise, in-vivo determinations of red blood cell recovery and survival through radiolabeling studies fail to measure oxygen delivery at the tissue level.

Current approvals for significantly altered and improved red cells rely very heavily on expensive, large scale clinical trials. While necessary at this time, FDA recognizes the need and desire for simpler, more expeditious methods of product validation. FDA acknowledges the need to examine the process for approval of new methods to apply to the evaluation of red cells. Innovations in the preclinical evaluation of these new methods for red cells are needed that would reliably predict clinical performance.

New pre-clinical markers will need to be validated in clinical trials and there will be the need to partner with the National Heart, Lung, and Blood Institute or other funding agencies to include marker validation in future clinical trials of red cells or to design and fund clinical trials specifically for validating potential markers. However, the potential benefit resulting from this work is significant.

So with that, I would like to wish all a highly productive and enjoyable workshop.

(Interruption)

DR. DOCTOR: -- it probably isn't, and we need to consider some complexities when we make our valuations. So, in the past, we generally thought, you know, the donor red cells is good as a recipient red cell, and in fact the main thing you need to be concerned about with a transmitted disease spreading about is known to (inaudible); in fact, it is unambiguous that donor and native red cells did not exhibit similar physiology nor efficacy. (Inaudible) that's also there, the

question is whether there's an impact that (inaudible) which is not particularly clear and how do we evaluate it.

It's also surprising that these differences can impair our oxygen delivery to tissue, which is (inaudible) with oxygen delivery, and it appears that (inaudible) with the way it interferes with homeostatic mechanisms, and it can not only not deliver oxygen as it is supposed to, but interfere with oxygen delivery by normal red cells but of use in the (inaudible) data. As a consequence when the harms are outweighing the potential benefits, some patients are getting hurt by the transfusion, and that's a surprise. So, the reason we are here, there's a sufficient basic translational clinical evidence of harm consideration of a fundamental change of blood banking and transfusion medicine.

So, let's start with the basics. Red cell function of oxygen transport is supposed to

move oxygen from lungs to aspiring tissues, and overall should be to improve that, but it's not simple. Unfortunately, the interaction here is fairly complex. So, I've organized my remarks in the following way. In review of the role of blood cells in regulating oxygen delivery, in fact, there are some fairly complicated roles that have to do with regulation of regional blood flow.

Some of the homeostatic mechanisms of oxygen delivery and the setting in of anemia, we'll discuss that, how storage lesion may interfere with this, the influence of transfused red cells, and the recipient O2 delivery in humans, and then if you think it's relevant to talk a bit about transfusion decision-making, because if we are giving blood to people who don't need it, it's not really the appropriate context in which to evaluate the balance of this in the blood flow (phonetic).

So we used to think of the role of the red cells and the wall that sort of partition but that's where blood O2 content resides, and the

HARP and the vascular tube responsible for moving the blood from the lungs to the tissue, and in the right efficient fashion. In fact, it's very clear now that red cells themselves are fundamental to this process, and they are interacting.

And so red cell based-signaling is fundamental for oxygen delivery in homeostasis at the cellular tissue and whole evidence level. And in fact, the first red cell based- signaling is somewhat new. When I was in medical school and we weren't discussing vascular signaling by red blood cells, although many of the people in this room unveiled that rule. In fact, I remember the first person to do it was Giden, you may have recognized the name, and, you know, the issue here is what governs blood flow distribution. There really needs to be a fairly efficient matching between distribution in both space and time (inaudible), as you exercise where your metabolic state changes blood flow needs to be redistributed in a fairly efficient way.

Now he showed this, that the (inaudible)

really resides in red cells themselves. This is an interesting preparation of the spinal animal, so he removes the influence of autonomic nervous system. This is a dog with a femoral artery and vein cannulated, and he's holding a flask blood up above it. It's fairly simple, a beautiful experiment, and he put blood of varying saturations in the flask up above the artery and then watched how fast it ran out.

And as he put progressively less well-saturated blood in the flask it ran out faster, and he presumed that red cells are offering, if there's a dilator, there's a function of desaturation. In fact, they do so in a way that perfectly stabilizes oxygen delivery; and that when the saturation falls below by about 70 percent that homeostatic mechanism fails, and oxygen delivery is impaired.

So, the importance here, it's that flow really trumps content, in terms of O2 delivery homeostasis. And that's the cue. So flow can transfer by logs in human physiology, content is

transferring really or manipulated in very small amount. This is interesting experiment to the (inaudible) looking at high altitude adaptation, they demonstrated that oxygen delivery to the forearm, really has almost nothing to do with hemoglobin concentration, but tracks almost perfectly with blood flow.

So, our cues for transfusion are hemoglobin, but the thing that we should be monitoring is flow or O2 delivery, so the content, per se, is not as important as the flux. And that's true in metabolic studies, but also true in human physiology.

So Jonathan Stamler demonstrated one system, there are several, but red cells do have context response of vasoactivity. This explains the findings in Guyton's dogs. As red cells are dropped in what's called the vascular ring preparation and the aortic slice like a loaf of bread, suspended, and when it constricts there's

an increase in tension, when it dilates it relaxes. Oxygenated red cells caused the vasoconstriction; deoxygenated red cells cause the vasodilation.

The blue blood put above the dog, in Guyton's preparation causes vasodilation increased flow. The red blood did not. This has been shown in other preparations; it is not an endothelial, epithelium-dependent, not an eNOS-dependent phenomenon. We were able to demonstrate that an S- Nitrosothiol is exporting red cells as a function of desaturation and can be captured outside the red cells, so there's an RDRF that's coming out as a function of vasodilation. But one in a thousand hemoglobins it's carrying NO, it's only about 450 (inaudible) are in blood, and it's fairly potent, about 1 percent released in the course of circulatory transit.

This serves to redistribute blood flow from areas of profusion excess to profusion lack. It's also been demonstrated to be fairly important when a residue in the hemoglobin beta chain is

deleted that's part of this system, then the mice are unable to support the increase in blood flow in hypoxia, in fact they have the opposite reflex. So this is a slightly more sophisticated way of showing Guyton's experiment.

In fact, when mice are exposed to hypoxia they have

(inaudible) changes, they have impaired physiology with their hearts, and in fact they have lethality. So this role for red cells is important in the physiologic response to hypoxia, redistribution of blood flow, and the ability to withstand hypoxic stress.

This, as I mentioned, is only one system, there are many others. Mark Gladwin and others have shown that hemoglobin can process nitrite and export NO as a function of deoxygenation, ATP can be exported as a function of deoxidation, and vasoactive lipids can be

exported as a function of deoxidation, but all agree that this red cells transit of hypoxic vascular bed, they export the vasodilator, they leave behind a bigger blood vessels than they entered, and so resolves profusion insufficiency. And if a transfusion is interfering with that physiology it's going to interfere with oxygen delivery.

So what do we know about anemia tolerance in humans? Weiskopf in the late-'90s showed us. So, he took some normal humans and brought their hemoglobin down from normal to about 50, there's an increase in heart rate, there's an increase in cardiac output, there's not much change in sort of feeling pressures, and there's a very significant drop in after-loads. So the point is, to get a vasodilated, hyperdynamic system, oxygen transport tails off a little bit, oxygen extraction increases, this is venous saturation. However, what's fascinating is oxygen consumption, actually increases a little bit as we get anemic, and there's no evidence of supply

dependency all the way down to a hemoglobin of 5.

And the key here is that oxygen consumption is going up, the ratio of oxygen consumption delivery is going up, so the cost is that the heart has to work substantially harder and is consuming more oxygen in the setting of anemia than not, so this gives us a cue that we -- our physiology here, our tolerance to anemia is dependent on robust cardiac response.

In children it's taking them down to 3, and there's still no supply dependency, the physiology is basically the same, there's a vasodilated hyperdynamic state, the extraction ratio goes up, and oxygen consumption goes up. However, underlying condition alters anemia tolerance, and the degree of loss resilience is condition-specific.

We are looking at about a 0.25 million patients here, and you can see that this is the odds ratio of mortality as a function of anemia, and you can see that whether or not you are old, have cardiac disease, lung disease, et cetera,

there's a difference in tolerance.

So, imagining that we have -- there's a broad brush and we should be giving everybody blood because their hemoglobin is 7 of 9, is a bit naïve. And imagining that we don't need to monitor oxygen delivery as we make our transfusion decision-making, is a bit naïve. And so we need to have an understanding really that is not only disease- specific, developmental and age specific, but (inaudible) recovery a full indication for transfusion might not be necessary, whereas when they are deteriorating, the same exact rate that I've indicated blood should be given.

So, I want to, again, frame this -- the clinical data and the physiology we are going to discuss in interesting construct. It has to do with hormesis, and really the potential sort of salutary effects of a little bit of anemia, and the physiology that's provoked, like poor oxygen delivery. So this is a plot that many are familiar with, you could think of this as a drug dose or, in this case, oxygen delivery or its

lack. The scenario of homeostasis, too little is bad, too much is bad, and we have sort of a Goldilocks phenomenon.

Now, you might say that for most things that we think of toxic, it's just -- a little bit is bad and more is worse. For example, cyanide, smoking, bullets, there isn't sort of a benefit from a little bit of that, however, there is a benefit from a little bit of oxidative stress, there may be a little benefit from a little bit of hypoxia in the right setting. You could think of think of this as red cell mass, you could think of this as oxygen delivery, you could think about this as tissue respiration.

So Risto has shown this, in fact that with calorie restriction there's a health benefit. With hypoxia, if signaling is initiated (inaudible) but has own ambiguous metabolic benefit, in fact, it's been exploited in terms of preconditioning in certain settings. So there's the possibility of a (inaudible) response, and if there's an area of homeostasis then it really

shouldn't be interfering with, with the transfusion. That's been unambiguously shown with the antioxidant therapy, antioxidant therapy is worse than no antioxidant therapy under certain settings.

There's been a recent review demonstrating not that recent, but a review demonstrating all the sort of salutary signaling that's initiated by anemia, these are all recognizable, too much is bad, a little bit may be good, because remember all this is occurring in the context of other disease. We are going to focus on the brain and don't look at the details, but there's obviously a fairly elaborate physiology here that stabilizes oxygen delivery to the brain.

In fact, the red cells are a large part of it, this is a more comprehensive demonstration of this these vascular signaling by red cells, and includes both the nitrosothiols, ATP, prostaglandins or signaling lipids, and also activation, hypoxic activation of nitrite. So

anemia and hypoxia induces salutary signaling that stabilizes both neurons and microglia in the setting of hypoxia. And it's been shown in a summary of transfusion trials in brain injury that really there's a benefit from transfusion when you are outside the area of homeostasis and (inaudible) blood transfusion when you are within the area of homeostasis, and we are indeed a third access coming in and out, and this relationship slides as a function of the level of the injury.

And really we should recognize that there's a condition of homeostasis where the physiology is appropriate, it should not be interfering with it, and we know that (inaudible) and homeostatic balance and the transfusion of (inaudible 00:23:17 to) where the implications are. And so I'd like to just now, just show you (inaudible) as many of you will be talking about this in detail, this is (inaudible) giving you some

fascinating talks about the (inaudible) metabolism.

In fact, there are some assumptions of (inaudible) and it's suggesting that, in fact, the oxygen (inaudible) distributed there at the location where the place of circulation where the oxygen is delivered the more hypoxic -- the more hypoxic tissue (inaudible). This generation of cytokines and bioactive reagents that interfere with stimulants that there's more in the bag than we expect.

And there's some (inaudible), and we won't discuss this in detail, but this is of course material flow, both aggregation, adhesion and inability to form is a problem, and obviously what I'm focusing on a little bit is the control of regional blood flow, and when the transfusion is interfering with normal metabolism, it's interfering with our ability to send blood where it needs to go.

So, if you look at the ECMO literature, and the ECMO is a heart-lung machine, there's

fairly robust transfusions occurring in ECMO. Now the thing is that there's no cardiac compensation in this study, so it's interesting to see what the benefit of the transfusion might have in terms of oxygen delivery, when cardiac output in humans is fixed by a bypass machine. And this has been done, and there are hundreds of transfusions in this data here, and if you prod the pre-transfusion venous saturation against the post-transfusion, venous saturation, there is no change.

If you brought the pre-transfusion tissue oxygen saturation against the post-transfusion tissue oxygen saturation, there is no change. What we ought to see is everything in this box full. So if someone has a problem with venous saturation it ought to improve with the transfusion but it didn't. In fact, if you look at this data again as a function of pre-transfusion hematocrit, it doesn't have an influence upon venous saturation, pre-transfusion hematocrit doesn't influence tissue oxygen

saturation. So transfusion, when cardiac output is stable, has no impact on oxygen delivery.

What you do see, however, is harm, transfusion or red cell utilization improves the likelihood of death, or increases the likelihood of death in ECMO in more than one dataset. So, if there's no benefit, there's only harm, and this is really one of the settings where it can be isolated.

However, it's clear anemia is bad. Dr. Koch has shown us in a series of nice papers, the anemia and the

(inaudible) when there's heart disease, so these are populations awaiting cardiac surgery, when the patients get anemic there's a series of poor outcomes, kidney injury, heart injury, you get stuck on a ventilator, you have a longer ICU stay, and you die. So, anemia is bad.

Transfusion also seems bad, as a

function of blood, mortality goes up and a series of morbidities that we are all familiar with. In fact, it's almost impossible to detect the benefit from transfusion, so the question of whether transfusion even treats anemia in this setting isn't clear. Now, of course they are fairly conservative that -- this is the Cleveland Clinic, these are the -- there's a frequency distribution plot of their incidence of transfusion, you can see they don't use a lot of blood.

But here is survival by years, out to six years. It's a function of pre-cardiac surgery hemoglobins, 25 percent, if they gave blood it was worse. If you didn't -- if your pre-cardiac surgery hemoglobin are -- as a matter of fact it was less than 25, obviously there's an adverse impact of anemia, but transfusion also makes it worse. So at no point, really, does transfusion improve your outcome in this setting. So we never really looked at that.

You are comparing doses or thresholds, and storage of blood, but whether transfusion

itself is useful, is something that isn't clear, and it's certainly clear that you shouldn't be making that decision based on the hemoglobin concentration. However, if you optimize hemodynamics, this is goal-directed hemodynamics support after cardiac surgery, and you improve blood flow, cardiac output and oxygen delivery through a series of interventions which stabilize the homeostatic system, and you only give transfusion at the end, you actually have an outcome benefit.

So, they tested this goal-directed response in a couple hundred patients, high-risk cardiac surgery. They did a series of interventions, no transfusions were required, versus usual care where they gave transfusions for a fairly conservative threshold, and there were some mortality benefit. Now, presumably, there is a level of anemia that does require transfusion, but it's pretty clear that it's not where we are currently transfusing.

So, what's happening in the bag with

regard to the systems that we discussed? We've been able to show that there's NO depletion, the ability to vasodialate and stabilize this hypoxic increase in blood flow is lost. In fact, importantly, it's lost in the coronary circulation. This is a study by Stamler Group in dogs where they are looking at flow in circumflex artery, it was cannulated and infused, either NO depleted or re-nitrosylated red cells.

And red cells that are NO depleted or re- nitrosylated, really don't influence coronary blood flow, when there's no hypoxia. However, the normal response when there's hypoxia, it's an increase in coronary blood flow of nearly 70 percent, NO depleted red cells can't do it, NO replete red cells can; so stored red cells can't support this physiology that's required. Weiskopf showed that we need to be able to increase cardiac output in anemia, and if a transfused red cell is interfering with that, we are going to have a problem.

Mark Gladwin showed this in a series of

very nice papers that this might be micro particles, now granted these are non-depleted add cell stored red cells, but you can see there's an increase in microparticles here. These microparticles can trap NO, and these microparticles cause hypertension. They also showed that these microparticles decrease in availability near the lumen, and in fact under conditions of flow, because the microparticles are streaming out into what's normally a vessel free zone, they are interfering with NO traffic between normal red cells and endothelium and really disrupting that response.

In a very nice set of experiments they show with acetylcholine infused into the brachial artery, there's a normal vasodilation response and that's an NO-dependent phenomenon, and when they then give blood, old blood interferes with that response. So, red cells can interfere with the vasodilation that's required for O2 delivery homeostasis. In fact, it's not just the NO trapping, but perhaps the arginase that's released

from decompartmentalized red cells.

So this is, again, the physiology that we are talking about, this is the supply dependency of oxygen so as - - this is oxygen delivery, this is really oxygen consumption, as you lose the ability to deliver oxygen you reach up -- there is homeostasis and you reach a point at which you become supply-dependent. We can also superimpose this area of homeostasis where, before you get to the area of supply dependency normal physiology is working fine, we probably shouldn't interfere with it, and if a transfusion is given here, we are going to -- in fact, you can push this point backwards, and you can create a supply dependency state as the data show from Gladwin, et cetera.

This is one assay, however, that I think is worth considering. This is a dynamic NIRS measurement, so most of the measurements that we make are static, if you occlude the brachial artery above the forearm, and you are monitoring tissue oxygen saturation, and you see -- you cause

blood flow cessation, you can monitor the desaturation. This slope here, demonstrates the relationship between oxygen consumption and tissue, and the oxygen content in the blood, and it's when it's released it shows the ability to vasodialate, and the ability to improve blood flow in the setting of hypoxia.

And you can monitor, in fact, this slope, and this slope here, and the return slope here indicates the ability of recruitable, so to speak, blood flow. So if cardiac output is poor, this slope is flattened, if oxygen content is poor this slope is flattened, if endothelial function is poor this slope is flattened.

Actually, there should be a slide that shows you that during transfusion, if you give blood only to those that have a flattened slope here, there's an improvement, and in fact that's the type of assay that we should be looking at, an assay that demonstrates an inability or lost homeostasis. So, if we use dynamic NIRS to monitor the ability to show recruited blood flow,

then we can make better transfusion decisions.

So I've tried to demonstrate that hemoglobin alone does not determine clinical severity and should not really be the key for clinical trial, but instead, you need to think about the magnitude of reduction of oxygen content, the change in blood volume, the rate at which these two factors occur, and the capacity of the cardiopulmonary system to maintain oxygen delivery. Really, the sufficiency of dynamic matching between oxygen consumption delivery, and this is what can be thought of as endothelial function, or the dynamic NIRS responsiveness, and can be impaired by microparticles.

One thing that should be mentioned is the sort of reserve inherent in red cell mass in improving oxygen delivery, even in the setting of anemia. These are the oxygen delivery curves of which we are all familiar. This is blood O2 content against (inaudible gap) with a hemoglobin of 7, this is the hemoglobin of 14. This is a normal, really, arterial PO2, and tissue under a

little bit of stress. That same amount of oxygen exported across that gradient.

This is the amount of oxygen exported across that gradient with a shift in the curve with a normal homeostasis inherent in the (inaudible) and the response to pH, DPG and temperature, et cetera. You can see that we really only lose a small portion of the oxygen delivery even when hemoglobin is cut in half. This is what we need to make up the cardiac output.

And it turns out actually that if you look at accumulative data from people who have refused transfusions, we tolerate hemoglobins down to 5 or 6 before there's really an increase in mortality.

So, how do we make the decision if it shouldn't be hemoglobin? This is a systems dynamic analysis of the transfusion decision, and really what we are balancing is the ability to tolerate a low hemoglobin against the influence of a transfusion and oxygen content which is

beneficial, a respiratory function which may be harmful, or cardiac output which may be harmful or beneficial, depending on context, and/or complications.

So, really, we need to think about it when O2 delivery is failing to meet metabolic need or failure is appending, or failure is sufficient magnitude to injure or threaten injury, and the risk exceeds the risk of not giving blood. And it's appropriately sequenced as is shown with the goal-directed approach and cardiac surgery. And then once the decision that transfusion has been made, a titrated approach needs to be used in order to give the least-effective amount, so that we do maximize the balance between efficacy and harm.

So how can you actually do this at the bedside? It seems overwhelmingly complicated. So you can think of this in three bins, nearly where there's compromised oxygen, or compromised reserve, there is O2 delivery homeostasis when we are approaching supply dependency but we are not

yet there, and the series of metrics that suggests we are approaching that point. When that's evident, compensatory physiology should be supported and oxygen delivery should be optimized and consumption should be blunted.

This is the goal-directed approach, and when that fails, anemia should be corrected. Likewise, if there's O2 delivery homeostasis and we are really in supply dependency, there's a series of metrics that might show that and we, again, support physiology, blunt consumption, and at the end getting transfused. Lastly, if there's no global problem, but there's a specific vital organ that's threatened, there's a series of biomarkers that are organ-specific, or their patients with known disease, and the same time support compensatory physiology to correct anemia.

And upon this approach it maybe more possible to see the risk, the relative balance of efficacy of transfusion rather than only giving blood to a hemoglobin where you can't really tell if they are getting -- whether there's a benefit.

So, that also seems a bit overwhelming. How could this be integrated in at the bedside? In fact, there are risk analytics or decision support tools that can do this; something that, perhaps, reports oxygen delivery effectiveness and integrates some of that physiology at the bedside.

So this can be modeled into a computer algorithm that integrates, has inputs from anemia, hypoxia and cardiac output, that is taught the relationship between these things and outcomes, and in fact you can program fairly complicated homeostatic physiology into this system, that then can be modeled and predict the hemodynamic response to anemia. In fact, this has been tested in children with cardiac surgery. This is the predicted SVO2. These are venous saturation as oxygen -- as cardiac output is fluctuating, these are the actual measurements and, in fact, look at the threadline, this is the likelihood that O2 delivery is impaired, and in fact the prediction of 02 delivery impairment does relate to outcome.

So, imagine a cue at the bedside that

indicates thread of oxygen delivery that can be a metric also that indicates attribution to anemia rather than problem with cardiac output, and that could be the trigger for a transfusion which might more clearly show the balance between efficacy and harm.

So, in summary, I'd like to say, suggest that red cells comprise the key node in the regulation of oxygen delivery, they match regional blood flow and tissue respiration, and participate in signaling that supports oxygen delivery homeostasis on a cellular tissue and organism level. The stored red cells strongly influences physiology and signaling and paradoxically they may impair homeostasis in a way that really we shouldn't be doing, unless O2 content really is the right limiting step in oxygen delivery.

And the transfusion decision-making, and in fact our ability to study transfusion efficacy requires a precise understanding of anemia tolerance that is the physiologic reserve supply dependency and specific vital organ threats that

is specific to various illnesses and their trajectory which also have a complex set of likelihoods from harm from transfusion.

And moreover, the sequencing of transfusion needs to be stable with other interventions that support oxygen delivery, rarely do clinical trials include such guidance, and our ability to monitor oxygen delivery components as well as the dynamic reflexes that comprise homeostasis really are required in order to make appropriate decisions. And this will enable titration of transfusion to the lowest-effective, least-harmful dose. In fact, until we have clinical evidence of blood that's being used appropriately, can we really feed back into the pre-clinical evidence of the quality of the stored product?

So, I do want to acknowledge the people who've generated much of this data, and with whom I've been collaborating. Thank you for your attention. (Applause)

DR. MARKS: Well, thank you very much

for that great overview, and if you are wondering about discussion, we are going to have a discussion at the end of the second session that will involve the speakers from the first session.

So, our next speaker is Dr. James Zimring. He is Chief Scientific Officer of Bloodworks Northwest, Director of Bloodworks Northwest Research Institute, and Professor of Laboratory Medicine at the Washington School of Medicine in Seattle. Thank you.

DR. ZIMRING: Thank you. Good morning. I'd like to thank the organizers for allowing me to speak today. I'm very excited to be involved in this process. I would also like to issue a personal apology. I've had a personal issue arise where I'm going to have to return to Seattle immediately after this session, and so I'm sorry for my absence, and I mean no disrespect to the other speakers. And it's sad for me, because it's really hearing what the other speakers have to say that I'm most interested in. But life gets in the way.

That was a great introduction, thank you to the first speaker because he was talking about the context in which we transfuse, and the variable and complicated landscape for which transfusion may be good, bad or indifferent. But we need to add to that paradigm, or that formula, that transfusion is not a monomorphic thing, that this is not a standardized drug where we can hold that as a constant variable, but the nature of the transfuse unit varies, it varies widely, and so you are giving different patients different things, and understanding what's in the bag and how it varies then is part of the equation moving forward.

So, I want to start just to point out, and obviously with my ambitious title, I'm going to talk mostly about other people's work, many of whom are sitting before me, and so that's a little bit humbling, and I hope nothing gets thrown. But the red cell storage lesions, obviously, has been appreciated for decades as accumulations of things that change as blood stores. And it's probably

the storage legions is more appropriate.

And there have been historical metrics that have been described. Changes in metabolites around ATP for energy generation, and DPG for oxygen association curves, numerous alterations in protein biochemistry that have been observed, redox biology seems to be very important, changes in cell- surface biochemistry which may affect how the cells interact with other cell types. And these include a number of usual suspects that have been demonstrated. Alterations in morphology, certainly as erythrocytes go from a nice biconcave disc to (inaudible), spiked beach balls, and so on that they pass a point and overturn.

And then also changes in rheological properties, and we understand that all these changes take place. What we don't understand is which of these changes have meaning and what context to the ultimate goal of therapeutic efficacy. So, we face three questions that I'd like to introduce today, clearly I won't answer them, but the first one is, how are patient

outcomes affected by difference in blood storage, whether they are a result of differences in donor biology, storage conditions or time, or as the first speaker really was introducing, recipient biology? Because, again, the landscape of what we are doing so diverse that asking simple questions of, is stored blood good or bad, is transfusion good or bad, becomes meaningless because of all the different categories we are looking at.

A second one, and I think more apropos to the purpose of this symposium is, what metrics can we use to predict the medicinal properties of a given unit of red blood cells prior to transfusion? This has both to do with licensing criteria for improved solutions, and for inventory management if we understand the different types of blood, or have different efficacies in different recipients.

And then lastly, is standardization of blood products a good thing? Or, are the differences between how blood stores its strength. Blood storing one way, and may have efficacy for

one disease, whereas blood stored another way may have efficacy for a different disease. And seeking the one, standardized immutable blood storage, one-size-fits- all, could be an error in that we could be throwing away certain therapeutic possibilities.

So, first of all, this question on patient outcomes: and I'm going to take us back more than a century to Ancient Rome where some of these scholars probably would have been the ancestors of Angelo. And refer to a fellow by the name of Galen, as we'd say in English, who was one of the most famous medieval or ancient physicians, he took care of the Pope while the plague was going through Rome, so someone was entrusted.

And he made a statement, and this is a -- you know, we can't hold them to current standards, but he made a statement which, in the context of how we now understand randomized controlled trials, it's comical and justifies us doing them. And the statement was, "All those who drink of this treatment would recover in a short

time, except those whom it does not help, who will die." It is obvious, therefore, that it fails only in incurable cases.

It makes a certain amount of sense, and if I can, for a moment, paraphrase and manufacture things that I hear from time to time, well, retransfused people don't store blood all the time, and people don't just drop dead, so we know it's safe. Sure, sometimes they die, but that's because they had other problems besides blood loss, and so transfusion alone couldn't save them.

I think this is a cautionary note to a type of thinking that we all do, and need to avoid, and it is precisely because of the randomized controlled trials, and prospective trials that are underway that will help us to avoid this trap. But it's a trap that we have fallen into and are getting out of and need to keep a careful eye on.

The point of blood not being a monomorphic thing, I think is made best by a couple of papers that came out in animal models by

Solomon et al. and Dr. Kline contributed to these, where in dogs, old or fresh blood were given to animals with different disease states and -- So, the black bars are old blood, the white is fresh blood, and then we are looking at survival.

And in the bacterial pneumonia model where the dogs are inoculated, and their lungs have a certain amount of bacteria and then transfused. The old blood causes death, it causes the bacteria to proliferate, aseptic asemia, et cetera. And clearly, in this case, the old blood is really a bad thing to give.

But, if you go into hemorrhage reperfusion injury, now the old blood is therapeutic, and it's the same old blood. And it's not hard to image how, if you stipulate for a moment that blood does accumulate pro-coagulant properties as we store it. For which there's a reasonable amount of evidence that it does, some of which we'll hear later in the seminar; that if you gave that blood with pro-coagulant properties to someone who was suffering thrombosis, you are

going to hurt them, and maybe kill them, but if you gave it someone who was actively hemorrhaging it's likely to be therapeutic.

So the question: is blood storage good or bad, is a linguistically meaningless question, when you understand that blood storage is multiple different things, and recipients have multiple different physiologies. And so we need to widen our gaze and our question to ask what's in the bag, and how might it be used to its greatest benefit in different context?

So, back to these questions, I want to focus on what metrics can we use to predict the medicinal properties of a given unit of red cells prior to transfusion, and really what can we learn about the quality of the unit, and the storage, both to guide us in therapy, but also guide us in development of blood storage solutions, and improvement there upon.

So now, we'll go to Medieval France, and a very famous case which illustrates the danger of having the wrong outcome. And I apologize for all

the words. So, Jean- Baptiste Denys, who was a French physician, and he had a patient, Antoine Mauroy, who was taken to running naked through the streets of Paris, a frenzy brought about by the mental anguish of a bad love affair. Simone, I think this is not so uncommon in this (inaudible)? Yeah.

But this is the state of poor Mr. Mauroy, and they wanted to flush the frenzy and evil vices from his blood with transfusion, which seems a reasonable thing to do. And they didn't want to use human blood, because humans are viceful creatures, you'd just be putting more vice on top of the vice on top of the vice and that would be bad. And so they used a calf because animals not having engaged in original sin, are viceless blood, and when they gave the calf blood to this gentleman the second time, there is now a famous description of what occurred.

As soon as the blood began to enter into his veins he felt the heat along his arm and under his armpits; his pulse rose, and soon after we

observed the painful sweat all over his face, his pulse varied extremely at this instant and he complained of a great pain in his kidneys, and that he was not well in the stomach, and that he was ready choke, unless given his liberty.

He was made to lie down and fell asleep, and slept all night without awakening until morning. When he awakened he made a great glassful of urine of a color as black as if it had been mixed with the soot of chimneys. And the problem here is not that we had just caused the first described hemolytic transfusion reaction. The problem here is that your observations are altered by your theories, because Dr. Denys was delighted at this therapeutic result.

Why so? He was delighted because he saw -- he observed the black urine, he interpreted it as a source of the patient's mental disturbance. A black color had been flushed from the patient's brain by transfusion. Clearly he had succeeded, and by his metrics the more black urine that came out, the more vice you flush from your patient,

and if he was developing blood storage solutions, forgetting for a minute that his patients would be dying, but by Galen's criteria that's okay, because they were destined to die anyway. He would be going for the blacker, and the blacker, and the blacker pee.

So, a slightly sardonic example, making fun of someone who lived several centuries ago, but the illustration that the endpoint that you measure will dictate what you develop, and be careful that you are measuring the right endpoint, which I'm not sure we are, and I think a lot of people here are not sure that we are.

So, there was a recent paper, which is really a pleasure to read, kind of a synapse, sizing where we are in this process and where we are going, and making the point that the FDA requirements currently are, and it's a little more complicated than this, but 75 percent red cell survival, 24 hours post transfusion, and less than 1 percent hemolysis in the bag, plus some other metrics.

And a very important question is, you know, first of all: How do we make blood from to comply better to these criteria, as we develop a solution? And, are these the right criteria to which we should be making the blood comply? Because if they are not the right criteria that we should be working towards, then what we are doing is making blood that makes more and more black urine, and feeling good about it.

Ernest Beutler, a famous biologist in many ways, but also in red cell preservation, made the statement, "No good surrogate test has ever been found for the performance of viability studies in human volunteers. Although the popular misconception persists that it is the ATP level in cells that determines whether or not they survive, this is a far from reliable indictors of viability, it is true that red blood cells with very low ATP levels cannot phosphorylate like glucose, and hence are fated to die. But high levels of ATP do not ensure survival of a stored red cell either."

This is a graph illustrating this phenomenon that I have pinched from one of John Hess' papers, and it demonstrates that, yes, at the extremes there's some predictive value here, but even ATP which is our -- you know, one of our hallmark, long-standing, this is what we measure, really doesn't tell us how well the red cells we are going to circulate post transfusion.

In fact, that's to the best my knowledge, there's nothing that we have that tells us how well they are going to circulate post-transfusion other than doing a chromium study, and even if that's the right endpoint. Now, let me take a step back. That's not say that there aren't things that tell us that red cells are going to do poorly. If hemolyzed in the bag, if the lactate and pH are way out of whack, if the ATP is way out of whack, we can tell you that it's not going to circulate terribly well.

But just because its parameters are good doesn't mean that it will circulate well, and we are kind of stuck and I think that's one of the

questions FDA and others want us to help answer. What is it we should be measuring; because if we are not measuring the right thing, we are working towards the wrong goal?

This is an example of a 51 chromium recovery, a recent one that was graciously given to me by Eldad Hod, and this is a typical kinetics that when you give a transfused unit of blood stored up to 42 days, you get most of the clearance within 24 hours, and then relatively normal survival, and that clearance can be about a quarter of the blood that you give to the recipient.

So if you give four units of blood to a recipient in a relatively short period of time, the particular endothelial system is consuming an entire unit of blood, biologically during -- in that context.

What I consider now famous graphs from a paper by Larry Dumont and Jim AuBuchon, corollary a lot of historical data, and storage solutions were changing a bit in this time, but looking at

the 24-hour recoveries across the population. As we observe is that the majority of people's store kind of around -- these are people tested, the majority of people stored test around this area, 75, 80 percent; some are quite remarkable. However, a percentage of red cell units are really rather horrible, and not just do they circulate very badly, but one could easily predict that they would have other drug in them that you wouldn't necessarily want transfused into your body.

It seems not such a big deal, that is a very small bar. However, taken into context of transfusing 13, 14 million units of red cells a year, into 1 out of every 70 Americans every year, having that small population of poorly storing blood suddenly, does not become so trivial. In fact, it might be quite important. Also, I don't think we know what's really going on, and so I'll tell you why. And so we discussed this with the speakers before the session.

To my knowledge the FDA guidelines for licensing red cells necessitates the labeling and

transfusion and studying of roughly 25 blood stores, give or take. So the first issue with is that of those 25 blood stores we are now going to study the biology and make an inductive inference from 25 blood stores to 6 million blood donors. So any statistician will tell you that assuming that 25 is a good representative example of 6 million is in and of itself a little bit dubious.

Understanding it costs a lot of money to do these trials, there's only so much we can do. But then there's a second consideration, because only 25 of them are studied, if one or two of them stores poorly, it can derail the entire product, won't make it to the market. Now, a lot of people participate in these studies repeatedly, and so when people are recruiting for these studies they often know who stored well in a previous study, and who stored poorly in a previous study.

So, I'm not going to make a statement, because I didn't do these studies, but I'm going to ask a question. And the question I would like to ask is, is it a correct statement that people

running the studies, preferentially choose those who have stored well in the past and ignored those who have stored poorly in the past, because doing so would favor making the FDA criteria? And if that is the case, are the 25 that we look at to represent the 4 or 5 million even less representative. Do we know? Do we really know how these solutions are performing in a broader population?

One of the problems in understanding the things that we should be studying is that we don't know why red blood cells are cleared. For those of you who like Thomas Kuehn and our post-modernist scientific philosophers who believe that the field has one paradigm, and then a crisis occurs and we shift to another paradigm, we are the opposite of that right now. We have 20 competing hypotheses, simultaneously, and they are data to reject and support all of them.

We don't know how red cells are cleared either in normal biology or in stored biology, and there's two basic camps. One is that red cells in

essence occurs and then the red cell simply accumulates certain changes such reticular and epithelial cells can eat them, and the various hypotheses are that red cell exposes phosphatidylserine on the surface, just the lack of ability to maintain membrane on asymmetry that CD47, which is a don't eat me signal, slowly decays over time, that the red cells have aggregation of Band 3, and thereby expose antigen to which naturally-occurring anti synesin antigen -- antibodies bind, and then together with FC receptors and complement fixation, opsonize the erythrocytes so that it gets consumed.

And so you'll see people measuring these things in- vitro quite frequently, but it's not clear that any of these is actually the mechanism by which the red cell is cleared in- vivo. It is very clear that if you take a red cell and purposely put phosphatidylserine on the surface, it will clear. If you take a red cell and remove salicylic acid from its surface it will clear. If you take a red cell and boil it in bleach, it will

clear, but because these things can cause clearance doesn't mean that's what happens in-vivo, and we don't yet know.

The other side, is eriptosis, so even though red cells lack nuclei and mitochondria, they nevertheless have the capacity to watch a Bears game and become despondent at the outcome, so much so that they commit suicide. They have within them the machinery to destroy themselves, and it typically has to do with a common pathway of calcium, influx into the cell, and then a number of activating events including exposure to phosphatidylserine, activation of caspase and calpain which then proteolyse inside their cell, and the circulation in the bloods that are unconsumed.

There's lots of noxious stimuli you can give to red cells and make them eriptose, and there's good evidence that some drug toxicities are a result of eriptosis, and that eriptosis itself may even be a defense mechanism against parasitic invasion by Plasmodium or Babesia, which

allows the red cell to get out of the way, and not allow more parasitic replication. However, it is stated quite frequently that eriptosis is how storage lesion cells get cleared, and it's stated kind of unequivocally in the titles of certain papers too, that storage of erythrocytes induces suicidal erythrocyte cell death, which may be the case.

However, there is a very important point to be made here. Is that if you look at these data, they look impressive that you start to get up to 42 days, and suddenly anexin positive erythrocytes, which is a reflection typically of phosphatidylserine externalization goes on. But in all of these studies what you'll observe they don't take the blood cells out of the bag, and stain them, they take the blood cells out of the bag put them into some other solution and put them in an incubator for 24 to 48 hours and cook them and then stain them.

True enough if you do this with fresh cells, you don't get this change. So, the red

cells have undergone some difference such that now when you insult them things go south. But this is a very scenario than you put them into a human and something happens. And we need to be mindful of the fact that we may search a bag of blood for this thing, that causes clearance, from now until rupture, and never find it because it might not be there. And why wouldn't it be there. It may not occur until the cells are reinfused, right.

And then they are circulating and you've got to study them. Now, there's another problem there, and as I say to all my graduate students who want open up a mouse and figure out why red cells are clearing by bleeding them, and I say, I'm sorry, but here's a logical certainty. If you are trying to study the thing that causes a red cell to be cleared, you cannot accomplish that goal by analyzing red cells that are circulating, because the thing that you want to study has not happened yet, by definition.

We can't reach a macrophage and get that red cell out, but it's the same thing with a bag

of blood. So, how might this be possible? Consider this: All eriptose -- All eriptosic pathways, of which I'm aware, necessitate calcium influx into the red cell. Citrate exists in our storage solutions to chelate calcium to prevent anticoagulation.

So, our blood storage solutions have in them, inhibitors of eryptosis. After you infuse in the body, calcium is again, plentiful, and then the cells may undergo the programming to divide, and so, again, looking in the bag may not be the correct place to look if this is what's happening, but it's not clear that we can measure these things directly in the bag and observe them.

This is not a new thing, so if you look at the text of Rous and Turner in their JX Med Paper from 1915 when they were first drawing blood. As experiments 4 and 5 show, these cells function -- they are talking about red cells -- normally even after they've been kept in-vitro fro -- my apologies -- for two weeks. We have performed a number of transfusions of cells kept

longer, they remain unhemolyzed for as long as four weeks, but by the end of the third week have largely lost their ability to be useful when introduced into the body, as shown by the fact that within a few days they disappear from circulation.

So, Rous and Turner were well aware of the fact, this is they had less glucose in these solutions, that red cells would preserve solutions can look real good and be great and there's nothing wrong with them until you infuse them. And then things go wrong. I wish I could write sentences like this. This is their next sentence, "The control rabbits all fared badly." I kind of like that very -- it wasn't rabbis, (inaudible) it's rabbits. So back to the future of RBC preservation, what Dr.

Beutler said was, "We understand much about how red cells metabolize under various conditions, but many of the advances in red cell storage had been the result of accident, or the

application of concepts that were later shown to be erroneous, thus we have stumbled not walked to bring red cell preservation to its present state."

I am hoping, with our modern advanced and ability and to test and observe, many of the leaders, of whom are in this room, that we will be walking now more than stumbling. So as we look at this historic list, we are now entering an age of ability to observe that far exceeds anything we could have imagined a decade ago. With the advent of metabolomics, proteomics, Mass-Spec-based technologies, advanced computing that allows the simultaneous accumulation of thousands of analytes in very small volumes of individual specimens, we can observe all components of the storage lesion to which we were previously blind.

This has made things much worse for the moment, because whereas a decade ago, there were 13 things we didn't know what they meant, there are 7,000 things that we don't know what they mean. But that's okay, because there are ways to figure out of the 7,000 things which of them may

be useful.

Rous and Turner describes red cells as bits of protoplasm without a nucleus, and if they are to be kept alive outside the body, there must be in what one might term a state of suspended animation. This is clearly not the case, this is -- with all due respects to eminent scientists, red cells are not in a state of suspended animation, unless the (inaudible) are all frozen. They are metabolically active, living things, undergoing a very strange environment.

So, Dr. D'Alessandro and other colleagues have made some very elegant analysis of the metabolomics of stored red cells and the phases that they go through during the red cell storage process which we now understand, and these have been very, very important observations. In my opinion though, these observations are limited to understanding the metabolic changes that happened with red cells as a group, and if the goal is to come up with new solutions or modify these biochemistries to make the whole group

better, there's a very rational excellent approach.

However, I think this approach is limited currently, in its ability to answer the question why do some units store well, and why do some units store poorly, and the only that's limited, is as of yet, I'm unaware, and I think some of these studies may be cooking. Of this analysis linked to a clinical outcome of the units so that you can do correlative analysis of the different pathways with known biological outcomes, as opposed to accumulating encyclopedic knowledge of what's storing which is clearly the first step.

So, recently the same group, a very nice paper in Blood, has ice -- has distilled these things down to 8 biochemicals that appear to be -- you can regress to, and appear to be amongst the most important predictors of storage lesion as you go along.

I, myself, have made the Faustian deal that I engage in experiments that are much logistically easier to run than human trials, and

in doing so sacrifice the necessity that why study actually correlates to humans. Although I believe in many cases it's likely to. What you are looking at here is a phylogenetic tree of mice, and understanding that all humans - - Well, that humans have differences in their storage biology, genetically, we grabbed the circled mice from around the phylogenetic tree, these are all inbred homogeneity, they all represent very small slivers of what mice may be, and started analyzing them for blood storage.

And we didn't -- We chose them not just because they were far apart, but also because the baseline hematological parameters were different, their reticulocyte counts and the hemoglobins, and et cetera. And in three of the three experiments what we observed is, under the same storage conditions some strains of mice store extremely well, and some strains of mice store not so well, some strains of mice store just horribly.

And these are 24-hour recoveries that we are doing, and it's a little bit different of how

they are done humans we are doing -- we are putting the whole unit into 24 recovery based upon it; and so to us this is a very exciting experimental framework upon which we can start to ask what are the differences responsible for how this blood stores differently.

And we took a metabolomics approach much like is being done simultaneously in the humans, and the first thing I can tell you is, and what you are looking at here is the -- the white is at the time of collection, and the grey is after storage, that glucose goes down in all the units, very much like human blood. 2 or 3 DBG, rapidly drops like human blood, lactate, accumulates like human blood, these are metabolically active cells, but the classic pathways of glycolysis which we study do not correlate to the post-transfusion survival in any of these animals, very much like the ATP levels in humans, correlate if at all, poorly.

What we did do is look through a great number of analytes, and with caution to avoid

errors of multiple observation bias, we stumbled upon a number of pathways which correlate very strongly. So, certain lipid metabolytes, in particular dicarboxylic acids, monohydroxy carboxylic cell acids, and heat metabolytes which are eicosanoids that come from arachidonic acid metabolism, gave us very strong predictions, you know, correlation of negative H7 with a P value of 8 times 7 to the negative 14th.

And so in animal models these just seems to be very robust and with LHON controlled pedigrees between the animal models and observe the same thing. So, here we have a linking of metabolomics to an outcome. Now again, whether that outcome is the right outcome for clinical benefit, we don't know. But this is the outcome that the FDA currently uses for licensing blood.

This is an analysis using a targeted lipid metabolics panel that we've recently developed, that can get human blood, and if you look at the same lipid metabolytes in stored human blood, they increase over time, just like they do

actually in mice. And so you can see the increase is predominantly arachidonic acid, and linoleic acid, et cetera.

And we have now analyzed about 250 donors of blood works, and not only do these things grow up in people but they vary pretty widely in people that are logged, and so this is a framework where we can now take people who have extremes, bring them back in and do chromium recoveries and see whether or not that predicts, or anon. This is obviously how one has to use animal models and it generates ideas and then you go forth and test them in humans.

I was very much intrigued by this paper by Palsson and colleagues on the eight metabolytes they identified in the human samples, and so when I saw that I immediately went back to our mice data, and looked for those same eight metabolytes in our comparison of different stains to see what they had to say about that.

But here is what the mice have to say. For lactate, malate, glucose, 5-oxoproline and

adenine, there isn't much there from the standpoint of those predicting post-transfusion recoveries. Now, I'm not telling you that those metabolytes don't change in the same patterns that they do in human blood, over time, but what I'm telling you is that they are not predictive of post-transfusion recoveries.

However, xanthine and hypoxanthine did have the appearance of correlations with low P values that would be as predicted, so as a survival goes down, with the xanthine and hypoxanthine. And this is not to be unpredicted because xanthine and hypoxanthine, and xanthine oxidase is one of the primary ways other than hemoglobin association that cells can generate reactive oxygen species.

So, as those things go up, the survival goes down, and it will be predicted that these might contribute to lipid oxidation which was the downstream thing that we were looking at. So, it's intriguing, again, to be going back and forth and asking these types of mechanistic questions,

and seeking points of specific intervention.

All right, so to kind of finish up, I want to go back to this 24-hour recovery which Eldad graciously provided, and asked the questions -- take the question even a further step back. Instead of asking, how can we figure out things that make 24-hour recoveries best? I'd like to ask the question, is chromium-labeled 24-hour recoveries the right thing to be measuring?

Now, clearly, clinical outcome is the right thing to be measuring, right. Clearly how the patient does is the right thing to be measuring, that's much more expensive diversion, and harder to get at, but what about this. So, Eldad did Chromium Survival Studies, but also transfused whole units of blood, right, because chromium, you have red cells, it's a small volume of red cells, you've watched them multiple times in the chromium labeling and thereby may have changed their underlying biology.

And then you are putting them in, and you have a decaying thing that you have to correct

for over time. He put in the whole unit and looked at indirect bilirubin increase which would reflect red cells being consumed by the reticular and the epithelial system and then metabolizing their hemoglobin products. And again, this presupposes that you have a recipient who does not have a metabolic defect in bilirubin metabolism.

But what he observed was something like you might predict. That over time, after transfusion, the bilirubin goes up. There is no radiolabel here. And serum iron goes up after around four hours, and then peaks because (inaudible) pathways kick in which stop its further increase. I don't have time to go into the details there, but this is what it looks like if you are looking at serum iron, transfusion of one-week-old blood does not increase serum iron, this is two weeks'-old blood, three weeks, four weeks, five weeks and six weeks.

So there appears to be this point, after about five weeks where you really get this increase in serum iron after transfusion, and also

bilirubin in these other pathways. This is what the bilirubin looks, they are very similar pattern.

However, when you look at post-transfusion recoveries with 51 chromium, the pattern, although there, is much less clear, so there's a significant overlap in the 20- hour recoveries between one and six weeks, when you look at chromium recoveries. If you look at serum iron, there is much less of that overlap, and it's more physiological in a way, because you are giving the whole unit and looking at its breakdown products.

And this is an area under the curve for non- transferring bound iron, looking at six weeks of storage compared to the rest, and again it gives kind of what one might predict. So, back to the final question, are we even measuring the right thing in the right way? And so, much attention needs to be given to that of course, because if we are following black urine as the thing we are trying to improve with our storage,

we are going to be modifying our storage systems incorrectly.

So those are my two cents, and I thank you all for your attention. (Applause)

DR. MARKS: Thank you very much for that wonderful talk. Our next speaker is Dr. Simone Glynn. She's the Branch Chief of the Blood Epidemiology and Clinical Therapeutics Branch, at the Division of Blood Diseases and Resource at NHLBI.

DR. GLYNN: So, good morning. It's a pleasure to be here, and my talk is going to be quite different from Jim. I'm going to be talking funding opportunity announcements, and scientific priorities which actually are quite similar to what Jim talked about for some of them. All right, let me see.

So, how do we establish a strategic research agenda for red blood cell transfusion at NHLBI? What we do is, we continuously monitor and identify scientific priorities, and we do that through review of the literature, attending

scientific conferences, but primarily, thanks to you as investigators, because you provide us input when you attend workshops, working groups, et cetera, in terms of what major scientific priorities we should pursue.

One of these efforts I'll just mention is also the NHLBI strategic vision plan that took about two years to be put together, and again was -- what happened is this was an effort where we asked for input from everyone from the public from the scientific community, they provided us with thousands of potential scientific priorities for consideration, and these were distilled down to about 130 scientific priorities which are in the strategic vision plan. And a little bit later I'll go over some of those that relevant to our red blood cell transfusion research.

So, what we do at the same time is we do a pretty routine basis, portfolio analysis to see what we are supporting in terms of research and resource, and then we put that with the scientific priorities that we know still need to be addressed

in the field, to evaluate essentially the gaps in research that we need to try to address. And then once we have identified those gaps we try to develop funding opportunity announcements around them, and the funding opportunity announcements is how we solicit grant or contract applications to address a particular research priority.

So, what I'm going to quickly do is go over six sets of major funding opportunity announcements that were developed in the last 10 years by NHLBI, to try to address, again, some of those gaps in our red blood cell transfusion research. The first one was an RFA that was released in March of 2008, and what we were -- it was a call for applications to evaluate the characteristics of our red blood cell storage lesion as well as its effect on the host.

So, this particular RFA was asking for a combination if you want, of basic preclinical and early physiological research. So, eight groups of investigators were funded in 2009, and have produced really, I think, a (inaudible) body of

literature which is really helpful to the field.

The second effort I'd like to mention is the RFPs that was a request for proposal that was released in 2009, resulting in the funding of the REDS III Program which is ongoing, and as part of this program, we are conducting a large study which is called the Red Blood Cell Omics Study, and Dr. Michael Bosch will talk to you about that. It will be later this morning, I think.

The third set of funding opportunity announcements, I would like to mention are the program announcement with review for transfusion medicine, so these allow you U.S. investigators to come in with either R21, which is a two-year funding period, or an RO1, which is usually four years of funding application, and then allows for the applications to be reviewed by a special panel that is put together with expertise in the field. The good news is that these PARS have been renewed just recently so that you can now apply, continue to apply to them until October of 2019.

The next set of funding opportunity

announcements I'll mention are the SBIR funding opportunity announcements that we had, we had two of them, one asking for research to try to essentially improve the storage of red blood cells through different technologies or strategies. And then the second one was asking for research to develop technologies to assess tissue oxygenation in a noninvasive manner.

And then finally, a quick mention about an ongoing program that we have that essentially is asking for our research to try to evaluate -- to develop high quality blood products from stem cells.

So, as we move forward, as I mentioned we, of course, still need to be very much aware of what our scientific priorities are, and what we did is we convened a state of the science in transfusion medicine symposium in 2015 to, again, evaluate what our scientific priorities in the field would be over the next 5 to 10 years. This was led by Dr. Spitalnik and Dr. Triulzi, we had over 300 attendees and I must say that most of

them really participated before the symposium in multiple calls to try to, again, identify and character the scientific, it was really a major group effort.

And the major areas that were evaluated were research on blood donors, research on platelets, plasma transfusion, and of course, finding out, what we are interested in today, the red blood cell transfusion.

So what I did put down is just some of the overarching themes that were identified at that symposium, and specific questions for the red blood cell transfusion research area. So I'll go quickly over them, and I'll of course encourage you to read the transfusion paper that was published in 2015 by Dr. Spitalnik, so that you can go into the details of those scientific priorities. But one of the major area of interest was that we need to do more research in terms of finding out what is in the red blood cell bag. So we need to identify and quantify the components of red blood cell products to improve the quality and

the producibility of these products.

The second major area was: what are the relevant red blood cell transfusion triggers, so is it hemoglobin or do we need to come up with another set of markers to better evaluate when we should transfuse? The third major area would be to do research to evaluate whether red blood cell transfusion works, and James certainly has gone over some of those scientific questions that we need to address. But essentially, we really need to try to establish appropriate physiologically relevant markers to determine the transfusion effectiveness, and probably do better than what we are doing now.

And then finally, how can we make better red blood cell products, and that's the question of how can we optimize or improve the potency and safety of transfusable red cells and for safety? The question of alloimmunization is a major one, how we can decrease that?

So, I already mentioned the strategic vision plan that was developed by NHLBI, but

essentially after the state of the science, again, where a lot of the scientific priorities were identified, you as investigators provided many of these as input into the strategic vision plan. So these were taken under consideration and thankfully some of them made it through in the final strategic vision plan, and I have kind of listed the ones that are of particular interest.

And so one of them was, again, what is the optimal red cell transfusion threshold, in both pediatric and adult patients? I just also would like to mention that we are funding a new trial, the Myocardial Ischemia and Transfusion Trial, or MINT, which is being led by Dr. Carson Dr. Brooks. And this is a trial that is looking at transfusing red blood cell at either a hemoglobin threshold of 10 or 8 in patients with acute coronary syndromes.

The second major priority that's identified in the strategic vision plan is how can we reprogram the immune system to improve outcomes of allogeneic cell therapies, and as you can see,

and also to diminish allogeneic responses to our essential biologic replacement therapies.

The third one is what we already working on, the development of safe, well-functioning designer platelets, and red blood cells from stem or progenitor cells. And the last one is, again, something that's very pertinent to our discussion today and tomorrow, is what technical improvements in the collection preparation storage and processing of blood products would improve their potency, safety and lifetime, and what biomarkers or other characteristics predict stability during storage and successful transfusion.

I also would like to mention that throughout those discussions, preceding the state of the science we also heard very clearly that there needed to be more research to harness or advances in Omics technologies and system biology approaches. That's important because it can fervor our understanding of red cell biology, inform efforts, again, to develop transfusion products from stem cells or their progenitors.

Better understand the effect of processing storage conditions and donor variability. Again, evaluate correlation between what's in the bag, and either 24-hour in-vivo recovery evaluations, or hopefully, maybe other measures that might be better or for cell effectiveness. And finally, evaluate novel additive solutions or storage strategies.

So, I'm not going to go over the next six slides, but I do have the -- they will be distributed to you, but essentially they contain the information on some funding opportunity announcements that are currently open that you may want to reply to. And then the last slide has some of our -- you know, some of us in our emails so that you can, please, contact us if you have any questions. Never hesitate to do that. Thank you. (Applause)

DR. VOSTAL: Thank you, Simone. And thank you very much for supporting research in this area. So, for our next speaker, I actually get to call my own number. So, I'm Jaro Vostal.

I'm at the Laboratory of Cellular Hematology, at the Office of Blood Research and Review, at the Center for Biologics at the FDA.

And what I'd like to do today is sort of lift the curtain to let you see how FDA evaluates red cell products. So as you can imagine some of the red cell products we get do have few blemishes on them from the storage or processing that they get, and our job is to use the tools that we have available to sort of identify the cells that we think will work as transfusion products or may not work as transfusion products.

So when the red cells come to us, they usually come in conjunction with other applications, and these are either for devices, drug solutions or standalone manufactured red cells. So for devices these are devices that would collect or process red cells for transfusions, these are submitted through the regulatory pathway of a traditional or de nova 510(k), or a premarket approval.

Now for drug solutions these are

associated with collections, processing and storage of red cells, and these come to us through new drug applications or abbreviated new drug applications. For manufactured red cells, these would be under biologic licenses application.

So I'm going to talk a little bit in more detail about the different types of applications that we get. So when it's a device-related red cell review, these usually are associated with apheresis instruments, automatic whole blood separators, leukoreduction filters, blood warmers, or similar types of blood bank devices.

The red cells are thought to be the output of the device, and are evaluated for their quality after the collection processing and storage. Now for devices, the review is risk based, and there are Class I, Class II and Class III categories that these devices fall into. Now Class I is minor risk so we won't really have to discuss that. Most of the devices we see fall either into a Class II or Class III.

So for a Class III device it's considered to be moderate risk. And if this type of device has a device that's very similar to it already on the market, that device on the market can be referred to as predicate device. Then with the predicate device you can compare yourself to the predicate and come in through the traditional 510(k) application. If there is no appropriate predicated, but the device is still considered to be of moderate risk, then it can come through the de novo 510(k) application.

Now for devices that don't have a predicate device, but are considered to be high risk, they go through, they come in through the premarket approval process, which is more extensive a review process compared to the traditional or de novo 510(k). When the device is approved or cleared for the U.S. market, the blood collection centers that distribute the products in interstate commerce, must then obtain licenses to produce these types of red cells.

Now for drug-related red cell reviews,

these red cells come as a part of a drug application for a blood collection or a storage system, these system usually consist of tubings, needles, bags, leukoreduction filters, and a drug solution. The intended use most often for these, is the collection processing and storage of red cells, and the drugs involved are frequently anticoagulants, additive solutions and processing solutions. And these are approved through new drug applications, or if they are generic drugs, through abbreviated new drug applications.

Now, for manufactured red cells, or red cell substitute, such stem cell derived red cells, hemoglobin-based oxygen carriers. These are going to be produced by manufacturers under a large-scale production, under good manufacturing practice with quality control and release tests. Now, at the end these manufacturers will obtain a biologic license application, a BLA to manufacture these products.

So this slide goes over the range of the -- the range of the red cell products that we

review, and it spans from conventional red cells for transfusions, and we think of these as the most -- simplest process to collect blood, manual collection, process was approved anticoagulants into approved storage bags, and approved additive solutions and stored under the usual temperatures, and for the usual 42 days post collection.

Then comes the slightly modified, a group of red cells, this is pretty much the bulk of the review process that we do that include the apheresis instrument collected red cells, as long as they have the same type of technology and same intended use, slight changes to storage bags, slight changes to additive solutions or leukoreduction filters.

Now finally there are the novel products that push the envelope in terms of state-of-the-art research, and these are significantly altered or synthesized red cells. Some of these include products that are chemically treated for pathogen reduction, ex-vivo stem cell, derived red cells, very extended storage, or

storage under unusual conditions.

So, the testing process that we recommend on these types of products depends on the difference between the standard red cells, and the new red cell that's being evaluated. The studies just briefly go into, initially it's in-vitro studies that evaluate the morphology, biochemistry and hemolysis of red cells, then we move to some Phase 1 and Phase 2 clinical trials, and these are frequently radiolabeling studies with chromium and indium. These are done in-vivos in healthy volunteers.

And then finally a Phase 3 clinical trial to evaluate the safety and efficacy of these transfused products. So, for red cells they have come in with minor changes, such as change to the additive solution or maybe an alternate supplier of raw material, it's possible that these could be evaluated only with the standard in-vitro studies.

For red cells that are a product of a more modified device, or associated with a more novel additive solution, it's likely that they

will go through the in-vitro studies, and then also be evaluated by some of the early clinical trials such as the radiolabeling studies focused on the kinetics, and particularly focused on the 24-hour recovery past transfusion.

Now for products that are significantly altered, are some of the ones I've already talked about, pathogen reduced, very extended storage, red cell substitutes, even the in-vitro studies are expanded to try to evaluate some of the lesions or issues that these products may have in comparison to our normal red cells. So, it's the more extensive in-vitro studies, also more extensive radiolabeling studies focused not only 24-hour recovery, but also on the survival of these red cells in circulation. And finally, it's very likely that these types of products will go through the Phase 3 clinical trial, and possible even a Phase 4 post-market clinical trial.

So, I'm going to describe to you in a little more detail, the in-vitro studies that we recommend for these types of products. We usually

ask that these studies be performed at two independent laboratories, and that's laboratories independent from the sponsor of the studies. And that testing be done at day zero and at the day of expiration of the product.

We ask for relatively straightforward results like cell counts, product weight, volume, hematocrit, and we also have some standard or some hard standards that we ask to be validated, such as less than 5 times 10 to 6th leukocytes, and the unit that's labeled as leukoreduced.

We also look at red cell, or request red cell morphology, MCVs, standard biochemistry tests including ATP, 2,3-DPG, glucose lactate, pH, PO2 and CO2. And we ask for free (inaudible) hemoglobin, and here again we do have a hard standard that the hemolysis level should be at less than 1 percent at the end of expiration or end of storage. And we also have several recovery standards that we apply to device processing, or red cells are frozen, thawed or rejuvenated, and these are 85 and 80 percent as labeled.

Now these studies come with some statistical considerations that drive the size of the tests, and for tests with the defined standard, like the leukoreduction standard, we require or we ask for a validation that the products have a

percent confidence, that 95 percent meet the specification. This is referred to as the 95/95 Rule. And under these requirements it takes 60 consecutive products with no failures to meet these criteria.

It can also prespecify a larger dataset that we allow at least one or two failures, but this needs to be ahead of the study, not after you've discovered certain failures in the dataset. Now for tests without the defined standards, such as the biochemical tests, we do a comparison to a conventional red cell product. And these red cell units collected by approved methods and equipment. And success in these studies is less than 20 percent difference between the value of the test and the control product.

And this should be done, again, with a 95 percent confidence that 95 percent of the products are within 20 percent. So, you may wonder where the 95/95 Rule came from, it initially was put into guidance in 2001 and it was the draft guidance for industry for prestorage of leukocyte reduction of blood components.

So those are the in-vitro studies, and now I'm going to move into the in-vivo 24-hour recovery of transfused autologous radiolabeled red cells. Now, these studies are performed under IND or an IDE for devices, they usually have

to 24 healthy volunteers. They are conducted in at least two test laboratories, and again, independent from the sponsor, and the criteria for success is a sample mean in-vivo recovery at 24 hours, of greater or equal to 75 percent.

The sample standard deviation should be less than equal to 9 percent, and we also have an additional requirement, that one-sided lower confidence limit for the proportion of red cell

components with a 24-hour red cell in- vivo recovery of 75 percent is 70 percent. So, this additional statistical criteria actually allows for low recoveries of less than 75 percent in 2 out of 20, or 3 out of

volunteers; 2 count of volunteers who actually may have some poor in-vivo recoveries on their own.

Now we do suggest that these studies also have a control arm, that means using red cells that are collect by an FDA-approved -- or these are FDA-approved red cell products, but this is not a requirement this is really a suggestion to be able to identify volunteers to come in with naturally low red cell recoveries.

So, over the years, this 75 percent has actually developed, and I just want to walk you through to the point where we are today. So, back before the late 1990s, these kinds of studies were done to support approval of red cell products, but they were done actually in a non-standard manner. They were relatively small studies, usually 4 or 5

volunteers, and so it's difficult to compare them to what we are doing -- to what's being done today.

So in the late-'90s we decided that we needed to standardize these studies, so we could do comparisons from lab to lab, and what we've settled on was the mean survival or great or equal to 75 percent, the standard -- a fixed standard deviation of less 9 percent, and a minimum size of 20 volunteers at two separate sites. And that's actually 22 sites, only 10 per site.

In 2004, we added this additional requirement, the one-sided lower than 95 percent confidence -- confidence interval for the population proportion of successes to be greater than 70 percent. And that allow for some of the additional failures in the study. Now, when this came out we did get some -- there was some concern in the field that products -- that this was too a stringent criteria, and that products in the field -- products already approved may not be able to meet the new criteria.

And we did take this issue to our blood product Advisory Committee in 2008, and we presented data that show that over the years, going all the way back to 1990 to 2007, these studies actually had an increased proportion of success over the time, so that in more recent years all the products that we on the market were able to meet these criteria. So based on these -- this historical look, we've actually kept the criteria in place, and its use on products that come to us today.

So, for additional -- For very novel red cell products there are additional studies that we ask for, and that's because these products generate concerns about potential toxicity and efficacy. So some of these issues that we are concerned about would be increased immunogenicity, reduced cell flexibility, increased fragility, low oxygen delivery capacity, and also unanticipated toxicities which we can't really predict based on just looking at the novel products.

So some of the studies that we think

would be helpful to address these issues are an extended in-vitro dataset that looks at oxygen dissociation curve, potential for 2,3-DPG regeneration. So, as you well know, 2,3-DPG declines during storage, and our question was, if the cells are treated, are some of enzymes inactivated so the 2,3-DPG would not be able to recover once it was transfused, and also for immunogenicity to detect any potential for a higher frequency of antibody generation.

And from the clinical perspective, these concerns continue so we have immunogenicity in antibody formation monitoring, the Phase 3 clinical trial for safety and efficacy, where they compare some to red cell products, and finally for issues that we don't think were picked up by Phase clinical trial, it would be a Phase 4 post-market study for very low-frequency adverse events.

So, in summary, our evaluation process is based on the extent of differences between a new product and a conventional product. In a

nutshell, highly different products get more scrutiny, the tests that include -- the tests include in-vitro biochemical parameters, and in-vivo clinical radiolabeling studies for from (inaudible), different red cell products, and significantly different product will likely need additional tests to evaluate red cell functions, such as oxygen delivery and safety in-vivo with animal models and clinical trials.

So, we know that the current review process that we have needs improvements, that's why we are here today. We are seeking input from the community as a whole to help us out, to help us redesign this process. Though some of the flaws that we think: is that the current process is designed to evaluate products that are similar to conventional red cell products, so we need to expand the process to better evaluate very novel red cells.

Another problem we see is that the in-vitro studies are not predictive, at least the ones that we have so far of clinical performance,

and so we need better preclinical tests that correlate with clinical outcomes. And finally, the in- vivo studies that we currently do, such as our radiolabeling studies, are really focused on red cell kinetics in circulation, but not on oxygen delivery. So, we need some preclinical and clinical methods to evaluate oxygen delivery, and we are looking for something that could be in-vitro or in animal models that could validated against clinical trials.

So, overall, that's our process, and we are hoping to get significant input from this workshop. So, thank you very much. (Applause)

All right. Our next speaker is Dr. John Hess, who is a Professor of Laboratory Medicine and Hematology at the University of Washington.

DR. HESS: I want to state that I have a conflict of interest. I am the inventor of Additive Solution Number 7, the patents are held by the U.S. Army and the University of Maryland, but I do get licenses royalties on them. And I have been a critic of the way the FDA regulates

red cell storage. Specifically for the best organization, I published a couple of years ago a paper on the scientific problems in the regulation of red cell storage, and specifically the issues that I am critical of, relate to the statistical models that we use to evaluate red cells.

As Jaro showed you, they say that they want 75 percent mean recovery, but will allow you 3 out of 20 products to be below that mean. What they are really saying is that you must have 83 percent recovery to pass their tests, and it would simply be useful if they say what they mean. Would

(inaudible) -- you know, good to approve Additive Solution 7, we ultimately ended up doing studies on 240 patients and recovery studies on about 54.

John Collins, famously said in 1973, at a meeting of the National Academy of Sciences, very much like this one, involved in trying to get adenine added to red cells. You know, the

experience at the end of the Vietnam War was that we had sent 1.3 million units of blood to Vietnam, had used 600,000 of them for a wastage rate over 50 percent.

It was possible to add adenine to red cells that had been demonstrated 7 years earlier. The Swedes were already doing it successfully, and yet it took another six years to get CPDA-1 licensed.

Red cells are the most commonly-transfused blood product, and as mentioned, we transfuse about 12 million products a year to about 5 million people. And that's about 35,000 units of red cells a day. And so that typically in the country there are several days supply on hand, this provides a fair buffer capacity for emergencies on either the supply or the demand side. You know, and when the electricity goes out in the northern states people don't collect blood, this can cause local problems on the supply side, and certainly when there are disasters or we have increased needs, there can be

demand issues as well.

You know, but the supply of group O and especially group O negative units is always a question. And there are problems with remote locations that need a few red blood cells. You know, as the blood banker in Seattle I have regional responsibility for trauma for approximately a quarter of the land area of the United States; Alaska, Eastern Washington, Idaho, Western Montana. These people evacuate their trauma patients either to us or to the Mayo Clinic, or to Salt Lake City, or Denver.

And so, trying to keep a few red cells in multiple locations across all of those areas can be extremely wasteful. And there are even places that are more remote. I was once the Director of Health of American Samoa, you know, which is five-and-a-half-hour air flight from Honolulu; Samoa is occasionally isolated for a week at a time in hurricanes, as happened in 2005.

And yet because all blood in the United States is tested for viruses that really aren't

efficiently tested in a territory of 30,000 people; you know, the blood is shipped from St. Louis by the American Red Cross, and they can be isolated, as I say, for a week at a time. Certainly when I was there I both had a bus go off a cliff, and had 30 people injured. You know, other kinds of injuries that used as many as 60 components, far more than they normally keep in stock. And we would have local blood drives, you know, and treat people with fresh whole blood.

There is now decreasing national usage, as you were all aware, and between the national blood surveys of 2011 and 2013, usage went down by 12 percent. A decade-and-a-half ago we talked extensively about the demographic bind, as half of the blood in the United States, the red cells are used by patients over 65, and their number was to double between 2000 and 2025. You know, we expected the need for red cells to increase by at least 50 percent. At the same time donor -- the age-specific donation rates were highly concentrated in the 45-year-old group, who

represented at that -- in 2000 baby boomers.

As those individuals aged and went from being donors to consumers, we've assumed there would be a large glut in our -- or a need for a additional donors. We've done a reasonably good job of both expanding the age range of donation, now a quarter of blood is collected from high school students in some areas, and certainly the acceptance of allowing older people to continue to donate, continues to improve. But, you know, we do need to continue to work on expanding the donor population.

Many people now follow transfusion triggers, and that allows us to donate less blood, and give it to people who are probably more likely to benefit from it. But the range of transfusion triggers in young and healthy adults down to 6 grams as recommended by the American Society for Anesthesia, for 7 grams trial trigger. You know, in most hemodynamically stable patients including ICU patients.

The active cardiac illness trigger of 8

grams as suggested in the TIGER-2 Trial. And finally a 9 gram trigger in patients in whom we are trying to suppress hematopoiesis in situations like sickle cell and unstable hemoglobinopathies in patients who have pulmonary hypertension, you know, allow us to have reasonable points to look at blood usage, and the appropriateness of blood usage.

There is at this point no really good evidence that long-stored blood makes a difference. We now have four randomized clinical trials that support that. This rather dense chart is blood usage at my hospital, and I suggest you go straight to the bottom line, where over the last 12 years, the number of blood components I am using is down by 65 percent. You know, we are at both historically low levels of usage, and low levels of wastage, and this kind of process saves $2 million in just the direct blood product cost, and many millions more in nursing time, and testing, and that kind of thing.

This is that same data broken down by

the individual blood components, for red cells, you know, the line is linear, for platelets and plasma there's a great drop off since 2008. These are the red cell data, you know, we built a transfusion service to break up the kind of monopoly that was had by trying to have a uniform transfusion service, that kind of had a vested interest in moving a lot of blood across town, and putting us in this situation where we sort were forced to transfuse blood because we had it.

But you will notice that blood usage has been -- decline has been steady. It's literally a straight line between 2003 and 2014. The early portion of that probably represents, you know, the rapid adaption of lower transfusion triggers in our intensive care units, the later portion of it, is probably largely reflective of much resuscitation policies that have reduced the total amount of blood use that we are doing since 2008.

We saw similar decreases in blood usage in the intensive care units at the University of Maryland when I was there. Here we can see a 40

percent decrease in total blood usage. The fraction of patients who got their first transfusion, add 7 grams of hemoglobin or below, increased from about 5 to about 60 percent during that time, and mortality in the intensive care unit decreased at the same time that blood usage decreased by 40 percent.

This is the data from the NIH-funded Glue Grant, 7 regional trauma centers that we are looking at cytokines in trauma, they discovered that the fraction of patients in their study who got one unit of red cells, which was the entry criteria, who went on to get 10 units, decreased by half as they began using ration-based transfusion triggers, or other transfusion. And this was associated with the decrease in the mean number red cells give to the trauma patients from about 6.6 to about 4.4, you know, about a third decrease in total blood usage, and the patients who actually got transfused had higher injury severity scores so they were using less blood to treat sicker patients with better outcomes, and

reduce total usage.

So, my hospital is now gone from using approximately units of red cells to 20 a day, and spend a great deal of

time doing things like improving our inventory management, and while we do not specifically care about the age of blood, in a recent retrospect a look at the blood use in the proper trial, our group, 87 percent of all of the blood that was given to our patients in the proper trial was less 21 days old.

This has an effect in trauma patients, simply in the fact that the increased use of younger blood means that relatively more of it circulates, and so we have more space in a sense, to give hemostatic products. We put blood on airplanes, you know, to try and improve the movement of patients in our very physically constrained, physical location, Seattle is within a few miles of very large mountain ranges, and getting people moved across Puget Sound, and down from Alaska, and out from Central Montana quickly

these distances are considerable, and so we put blood on these aircraft.

I would mention that the U.S. Military uses about 500 red cells a day, to provide them to the fronts, the range all the range all the way from the Korean DMZ, and the West to Afghanistan, and the East. And there are simply high rates of nonuse. We sent 6,000 units of Blood to Bosnia, and used 111, for less than 2 percent use rate. You know, blood that's stored two weeks longer, would have allowed us to do that with 2,000 fewer units of blood increasing the use rate to 3 percent but saving a great deal of product. There is a continuing need for more durable blood products in all categories for remote locations and military use.

Frozen blood is also clinically used, mostly to support rare donor systems, costs about four times as much and is associated with about a 20 percent additional loss. It's licensed currently for 10 years, and it's been demonstrated to be effective, you know, for as long as 37

years. And the Dutch and Czech militaries were able to use it almost exclusively in Afghanistan, you know, under the situations where they were using about 60 units a month. It was efficient.

It is possible to make better red cells. This is the recovery data, from CPDA-1 back in 1979, the licensure study. You know, the cells stored in CPDA-1 its whole blood are good, but when you remove the storage -- the albumen and the plasma and platelets, the storage falls, just because there's no place for the protons that are manufactured to go. When you put that volume back, it's an additive solution as shown here in the licensure study for AS-3, you can markedly improve that storage, but at seven weeks it does not work.

You will, again, notice the very large individual variability of donor to donor, that are seen in this. The standard deviation of the actual chromium test is about 4 percent, but the donor-to-donor variability is much higher. The Dumont and Canceles Studies, or the AF-7 Studies

at six weeks, stored with eight weeks of warm storage or 24 overnight hold, which would allow manufacturing facilities to get rid of their evening and night shifts. The solution is still robust out to about eight weeks, and has been licensed in Europe for that period of time.

So, you know, we do want red cells to remain available, safe, effective and cheap, and not irrevocably wrapped in red tape. The AF7 solution contains only more phosphate and a little bit of bicarbonate things that we already give in far higher doses to many, many people. And yet, you know, we took 11 years from demonstration to license

(inaudible gap) expensive and difficult process to do something that as clearly designed, you know, not raise any toxicity questions. Thank you. (Applause)

DR. VOSTAL: All right. Thank you very much. This brings us to our first break. So we have 20 minutes.

(Recess)

DR. SPINELLA: We're going to try to stay on time. It's a pleasure for me to moderate this next session. My name is Phil Spinella. I'm a pediatric intensivist at Washington University in St. Louis. I want to thank you all for involving me in this process. It's very exciting to see where this can hopefully go in the future. And it's a distinct pleasure of mine to introduce Dr. Harvey Klein. Harvey is the chief of the Department of Transfusion Medicine at the NIH Clinical Center, and he'll be speaking to us about the evaluation of red cell products for a transfusion.

DR. KLEIN: Thank you, Phil. I'm not going to be talking about how you transfuse or when you transfuse blood, although I agree entirely with Alan Doctor's comment earlier about the transfusion triggers. I generally refer to that and to some of the trials on which we base our triggers as imprecision medicine, but that's another talk for another day. So what I'm going

to be talking bout in a brief period of time is what's in the bag. And I think what we're looking for are markers to help us maximize red cell efficacy, to minimize red cell toxicity, and to ensure red cell availability. Since some of the earliest transfusions, this is appropriate for the week where Nobel prizes are awarded, Alexis Carrel was awarded the 1912 Nobel prize for anastomosis, which led to George Crile and other's publications of vein-to-vein transfusion. And here I don't think there was any issue about the quality of the blood. It was pretty much the same as it was in the donor when it got into the recipient, but you couldn't do too much with that. Unfortunately, for the vascular surgeons, though we'd have far more of them today if we were doing 13 million transfusions by the Correl method.

So it was really another Novel prize winner, Peyton Rous, with Rous-Turner solution, who added citrate and dextrose and, as Patrick Mollison said, separated the red cells, both in time and in space from the donor who made possible

the earliest blood banks or blood depots used by Oswald Robertson in World War I. Now, Robertson didn't do a lot of quality assessment of the red cells. They weren't stored for long periods of time. You can see they were in bottles and in cases. But they seemed to work. And when I say seemed to work, I think it was fairly obvious that in this particular population, young men who were exsanguinating from various wounds, those transfusions saved their lives. But subsequently, as you've heard earlier, and we'll hear later in much greater detail, we've appreciated that the longer you keep the red cells in a bag, in a refrigerator, the more things occur. There are metabolic changes. There are changes in shape. There are changes in membrane, their release of various kinds of small molecules, none of which are likely to improve the quality of the red cell, but the big question has always been, to what extent is this deleterious? And so when we think about the issue of red cell efficacy, as you've heard earlier, we're generally thinking about

oxygen delivery. And I guess that's appropriate, although the red cell does a lot of other things. It removes carbon dioxide. It binds nitric oxide in a variety of places. It binds cytokines. It has a normal hemostatic function and it probably has a pro- thrombotic function when it's stored for long periods of time. And then there's et cetera. But we don't really look for markers for these kinds of things. I think what we've been looking for is some kind of marker for oxygen delivery. And I suppose that's appropriate, but perhaps we shouldn't forget some of the other functions of the red cell.

And while I'm on the issue of efficacy, we are thinking about what's in the bag as functioning the way red cells function in our body. Changing them, for example, by storing them for long periods of time as we did in the dog model that Jim Zimring showed you, where the cells seemed more effective in hemorrhagic shock, that's a new component, really. That's not what we're looking for. We're not looking to modify the red

cells. At least for this symposium we're looking at the native red cells. And what we've traditionally used as a surrogate for oxygen delivery is that the cells are alive and circulating, and it's chromium-51 survival and recovery.

In terms of toxicity, there are lots of things that cause toxicity, the metabolic and rheological derangements, cell-free hemoglobin, nitric oxygen scavenging and release of iron have sort of been what I call the big three, but then there's an et cetera, et cetera, and et cetera. And again, how do we measure absence of toxicity or limitation of toxicity? And these, again, are surrogate evaluations, and they're essentially hemolysis in the bag, and again, recovery and survival. If you recover them and they survive reasonably normally, they shouldn't be toxic, I guess.

Well where does the issue of recovery and survival come from, and I'm not sure who the first one was to do this, but Patrick Mollison in

one of his earlier publications, Loutit-Mollison, with acid citrate dextrose, pointed out that, in assessing the preservative value of these and other recommended solutions, the chief criterion adopted by whom and why was the survival in vivo of transfused red cells which were stored in various solutions, and he points out that his solution is better than those that came previously by this criterion. But this was a relatively subjective selection because, I guess, there was nothing better at the time and it has continued for many, many years with the sole advantage, I guess, now, of using a standardized method for measuring. Mollison used not chromium-51.

So the current goal standards, as you've heard, is 75% of cells circulating at 24 hours at the end of storage, and less than 1% hemolysis. And there really aren't any requirements for standard red cells for clinical studies, and I would suggest that maybe there shouldn't be. These are very hard things to do, and we're never sure, given the heterogeneity of patients, what

they actually mean. Where did the recovery come from? Well, from the 1940s to the 1980s, subjectively on studies done by Joseph Ross and Clement Finch, 70% recovery was what we believed was sufficient. And then in the 1980s, again, totally arbitrarily we came up with the number of 75%. Is that the right number? I don't know. Is it an important number, 25% of the cells are dead on arrival? I don't know that, either. But, of course, there are a whole host of other studies that you've heard about that since CPD was licensed in 1957 are kind of routinely studied.

Red cell ATP concentration, as you heard, there aren't any set standards. And the correlation with in vivo recovery survival hemolysis varies among labs and isn't all that good to start with.

The oxygen dissociation curve, the equilibrium binding curve, and I'm going to come back to this in just a moment, but it's not simple to study. Everyone does it, probably, in a slightly different way. There isn't any great,

reproducible method of measurement, and there's always the question of clinical relevance. 2,3-DPG, again, we don't have any standards. And then there are a whole host of other things that are required, and they probably correlate, to some extent, with damage to the cell. But are they really important in terms of clinical outcomes?

Now, you've seen this slide already, really. These are the recent data that FDA suggests are necessary for a red cell storage, and it's what they're currently using if you come today for a new solution or a new bag to store red cells. You've also heard about the statistical considerations, and certainly it's very important that all of this be based on the best science and the best statistics, but the statistics aren't complicated, and they do, in fact, in some instances, stand in the way of getting the kinds of licensure data that perhaps would be relevant to outcomes rather than to simply a large number of statistically studied procedures.

Both radiochromium recovery, survival,

in red cell storage shows substantial donor-to-donor variability. And you've seen these slides before. Not this one. This is one that goes back to the '60s, 27 volunteers showing that some donors store very well, some donors store very poorly, and that's really quite reproducible. There are good storers, there are bad storers, there are average storers, and we're not entirely sure why that is. And then what I really do consider a landmark publication by Dumont and AuBuchon shows the distribution of red cell 24 hour chromium labeled recovery in different donors. And I'd point out just a couple of things. The first is that if you store for 42 days, and all of the data that were in the literature, the distribution looks something like this. But if you irradiate the cells, the distribution is somewhat different, isn't it? And when we license red cells, we license them for storage, I guess, but everybody irradiates them, so maybe we ought to know something about that, as well.

And again, when you freeze and thaw red cells, again, the distribution is again different than it is for just the stored 42 days. And even though they're licensed for storage in the cold, people do freeze thawed. We need to know about that. Is it important that different people store differently when their cells are frozen and deglycerolized? And then there's the whole blood oxygen dissociation curve, the respiratory function of blood, and you've seen this previously and it's required for all license applications, I guess. The curve shifts to the right with DPG and it shifts to the left as DPG is depleted and changes in pH and changes in temperature and how important is that?

Well, I'm just going to show you some very old studies that we did back in the '80s in patients with sickle cell disease. And I show you this because the first automated exchange transfusion in sickle cell disease patients was carried out in South Africa, and the patient who was exchanged rapidly became comatose. And the

publication says one should never ever do that because the dissociation curve shifting to the left doesn't delivery oxygen to the brain. Well, we decided to study this at NIH back in 1980. Dr. Robert Windslow and I took 10 patients and we rapidly exchanged transfused them, and did, in fact, see that the dissociation curve, as we did change of red cells went to the left. The patients, by the way, none of them became comatose.

The other part of this study was to look at their outcomes in terms of their physiology. So we kept their hemoglobins the same. You can see that here are the exchange hemoglobin As versus the pre-exchange hemoglobin As. The P50s came down as I showed previously, but surprisingly these patients had an improved anaerobic threshold when exercised on a bicycle, a stationary bicycle, prior to and following exchange transfusion. And the amount of work that they could do at a standardized pulse of 170 was dramatically improved. So their function improved, despite the

fact that their dissociation curves suggested they weren't delivering oxygen, as well. And this is just two of those patients showing on a bicycle ergometer, at the same level of work, post-exchange transfusion they had a lower heart rate at every level at the same amount of work, and the anaerobic threshold shifted to the right, meaning that they had a better -- they could do more work before they went through anaerobic metabolism. Patient number two shows the same thing as did the other patients.

Now, I'm just going to close by saying that we do need outcomes, and perhaps animal models, we're going to have two sessions on animal models. We'll be able to tell us what kind of pre-clinical data these would help us with, but it was interesting to me several years ago when we looked at this that of four different animals, and the fifth being man, if you looked at the VO2, in terms of their hemoglobin, and if you corrected for the differences in hemoglobin to start with, at about 25% of their starting hemoglobin, the

oxygen consumption fell off dramatically. So perhaps at this point all of the various compensatory mechanisms we've heard about are no longer functioning and maybe we could test the quality of red cells in an animal model in this way.

So how would I summarize the evaluation of red cell pleuritis for transfusion? First I think it's obvious that evaluation should provide a reasonable level of assurance of both efficacy and safety. The criteria that we're currently using, although somewhat arbitrary and flawed, have served us pretty well. And so if we're going to change them, the changes really need to be evidenced based. If we're going to go to biomarkers, pre-clinical biomarkers, they need to represent -- they reflect either red cell function or clinical outcomes or ideally both. The assays need to be reproducible and the statistics need to be achievable. And finally, the ideal evaluation criteria, and the appropriate statistical treatment, and neither currently identified nor

intuitively obvious, because if they have been, we would have adopted them already. Thank you.

DR. SPINELLA: All right, our next speaker is Dr. Jason Acker. Jason is the senior development scientist at the Canadian Blood Services, and will be speaking to us about predictive clinical value of in-vitro measures of red cell quality.

DR. ACKER: Good morning and thanks, Phil, for the invitation to come and present to you some of our data. And while I'd like to tell you that I've got all the answers north of the border, unfortunately I don't, so bear with me.

So in preparing for this I had the opportunity to sort of reflect on what my perspective of quality was, and I went back to some of the earlier work by Claus Hogman and Harry Meryman and quite eloquently in a review where they were trying to make the case for standardization of red cells, they made this quote, we're really tried to articulate what it is that we're trying to do when we actually transfuse

blood. I'd just like to point out that the physicians are assuming that us in the blood bank are actually giving them something that is replacing something that their patients are actually using or actually need, and I sort of query the question of whether that is actually being achieved in the current context of blood banking and blood transfusion medicine.

So I'm going to talk about hemolysis for the next few minutes and not because I think hemolysis is that endpoint measure that we really want to be measuring more of, but more because it gives us the opportunity to look at some data in a way that may help us understand what's really going on in the blood products and what it might actually mean to patients.

So many of us in Blood Bank know that hemolysis is one of the things that we look for when we're visually releasing blood products to the transfusion unit. But it's also something that within the context of the regulatory environment, it's something that we're measuring.

And hemolysis really reflects the fact that there are red cells that are old and they will break down and they will release the iron, free iron and free hemoglobin, into solution. And the body, naturally, has ways of compensating for that and accumulating that in normal, healthy individuals. But what happens when that occurs in the blood bag or in the blood manufacturing environment, and ultimately what happens when that occurs in patients?

So in this, you know, we study hemolysis really as an endpoint to the storage lesions that we've talked about and have been introduced already. And I won't go that into a lot of detail, but there's a lot of things that we can measure in the lab that contribute and correlate to that ultimate release of hemoglobin into solution. And if we measure hemolysis as a function of storage time, as many of us do, it increases with storage, ultimately exceeding some level at some time point in the future.

Now, one of the questions we had was,

what happens when we actually manufacture products using different technologies? So this was work that we were fortunate to do at Blood Systems with Philip Norris in San Francisco. And there we had the need to try to understand what hemolysis meant in Canada by going out and seeing what hemolysis was like at other blood systems. And many of us in the audience will appreciate that there are a variety of different technologies that are used to manufacture a red cell component. It can be whole blood derived using whole blood filtration type technologies, or it could be a buffy coat manufacturing method like is used in Canada. It's started to be seen here in the U.S. but predominant in Europe or, obviously, aphaeresis technologies. And each of those produces a red cell that, you know, in many cases, equate to being equivalent from a clinical perspective, but is that truly the case? So what we were fortunate to do is actually have all of these different manufactured blood products shipped up to my lab in Edmonton, and we actually tested them in the

same lab using the same diagnostic platforms. And one of the things we measured was hemolysis.

In Canada when we started this work we had a hemolysis standard that said, blood products had -- in 100% of the blood products manufactured, the hemolysis had to be less than 0.8%. And when that standard came out, those of us in the blood bank sort of shook our head and said, well we're not going to be able to achieve that. But the, you know, the regulatory agencies persisted and said that, you know, we should be able to achieve it based on the average, the mean.

So we went about actually measuring a lot of products in Canada for hemolysis to try to really understand what the true value were. And as part of that we looked at extending south of the border. And what you see here is that depending on the manufacturing method, at either fresh, which we equated as day five, and expiry at day 42, there's differences in the amount of hemolysis that's present in those products. Not surprising the non-leukoreduced product that are

available still in the United States have a high level of hemolysis in the product. But surprisingly, what we saw is that the aphaeresis technologies, again, give a higher level of hemolysis than one would expect in a whole blood derived product. And that's likely due to, again, differences in technologies and differences in how they're processing. But, you know, it was obvious and it was significant.

So the other question that we had was really what's our donors doing to contribute to this? So this is work that we've been doing closely with the Mark Gladwin group and Tamir Kanias who was my PhD student who's now a research associate in Pittsburgh working for Mark. Where we are really asking that question, what is the age and gender of the donor do? Really because there was some observational data that suggested that donor factors may be contributing. So one of the simplest things that we could measure, looking at our quality control data, was the effect of age and gender, so this age of donor, the gender, was

the actual effect on storage hemolysis. And, lo and behold, we showed what others have also showed now, that female's red cells hemolyze less than red cell -- male red cells in all test groups.

And if you look at that by blood manufacturing, what we also show, and in the more details available in the references, is that it also depends on the manufacturing, so there's a compounding effect that if the female blood is processed using one method, you get a certain level of hemolysis, but if you use a whole blood filtration process, you get a different level of hemolysis. So there's the interaction between those different variables. And we've been dedicating a lot of time over the last few years to try to understand why.

Now one of the things I wanted to have the opportunity to emphasize here is that the -- we talk about the standards that we should have and the 95 and 95% rule here applies in the United States. In Canada now it's similar. We've adopted a similar standard. But what a lot of

standard setting organizations don't appreciate is, it's one thing to have a standard, but you should also be commenting on how that method is actually to be performed. And I think Harvey just made the point in one of his last slides was that, we need some standardization in the methodology and to prove that point, what we did was a study where we looked at a variety of different ways that you can actually measure free hemoglobin and hematocrit and total hemoglobin, everything from automated technologies right through manual Drabkin's, spun hematocrit- type technologies.

And we looked at the effect that something as simple as how you centrifuge those samples prior to doing the analysis could have. And we combined all those variables, and lo and behold we show that for that exact same product that's tested using a variety of different technologies, you can actually have a 50% difference in your reported level of hemolysis, which was shocking that, depending on what methodology you're using, you could actually, you

know, select the level of hemolysis that you actually get. And, in fact, when we went and surveyed BEST members, we found that there was significant variability across the planet in terms of how that hemolysis test is being performed, to a point where I can tell you which blood systems have good levels of hemolysis based on the methods that they were using, and ones, like ours, the Canadian Blood Services, where we tend to be, I guess, more on the higher side for a variety of different reasons for why we chose to do that method, that our levels f hemolysis in our blood products will look worse. Does that mean that our two different blood systems are producing different quality products? No. It means that we're using different analytical methodologies.

So within Canadian Blood Services, within our quality monitoring program, we've developed a lab in Edmonton that actually measures a variety of different things for a variety of different reasons. And this isn't to show that there's a lot of things that you can measure. We

all know that there's a lot of things to measure. The point I wanted to make here is that we do have a lot of tools that when we have specific questions about asking what's happening to the product that's in the bag, we can select from a large number of analytical methodologies to try to answer that.

The ones highlighted in blue are the ones that our quality control program actually measures routinely on products through our manufacturing. I just wanted to point out that most of them are clustered in the unit characterization level. There are very few things that we do in quality control in a blood bank that actually measure quality. They measure characteristics. They don't measure much about quality. Hemolysis is the one exception that we routinely measure and we're regulated to measure on our blood products, and it does actually tell us something about the quality of that product.

But there's a variety of other technologies that we're using, because one of the

questions that we're asking, as this relates to the effective age and gender, is what populations of cells are actually in that blood bag? Because there is a lot of emerging evidence now to suggest that there are actually subpopulations, and I'm not talking about reticulocytes or nucleated red cells, I'm talking about red cells with very different physical characteristics that we would want to actually look at because they will respond to different manufacturing processes or actually different clinical scenarios, perhaps differently.

So we're using a single cell technology like the ImageStream X. We're looking at, you know, characterizing particle size using the Izon qNANO, but then we've also developed a number of micro phyletic technologies in collaboration with Stanford, but also in collaboration with a number of other universities. We're actually able to look at those individual cells within the bag of blood to try to understand what that means.

Now, depending on what you actually measure, you can actually get -- you can see,

probably, a similar effect. And this is data that came out of the collaboration that we've done with Philip Norris's group at BSRI, but everything from hemoglobin to residual plasma, to the residual leukocytes to the hematocrit, to extracellular vesicles. It doesn't matter what you measure, the point is really the same, is all of these differences are differences in manufacturing methods that are used. And it shouldn't be shocking, but sometimes people find this shocking, is that even something as simple as hemoglobin, how much of hemoglobin is in that bag of blood that we're transfusing? You could have a difference between

grams to almost 75 grams. So when we're doing transfusion trigger studies, and

we say that we're going to do a liberal or a conservative transfusion strategy, depending on where that blood's coming from, you may have a difference of 25 grams of hemoglobin in that bag. How can you do dose studies when you don't control the dose? So I find that quite interesting that,

you know, we have this conundrum in the field where we try to do clinical outcome studies when we don't know what we're actually transfusing.

It doesn't matter what you're testing. You know, all red cells that we look at meet the basic QC criteria. They are available on the shelves in the blood banks around the world right now, but they're different. And they're different in a variety of different ways that we can actually measure. They're not equivalent. So to expect that them to have clinical efficacy, the same clinical efficacy, is a -- it's absurd. We can't make that claim. So this isn't a call to standardize. Actually I appreciated the call or the comment from one of the other speakers is that, we have the ability through manufacturing to produce a product with a specific characteristic. What is that characteristic that we want for the recipients that we're studying?

So my group has been working with others to try to answer this question, do donor and manufacturing variability that we can actually

measure in the lab in-vitro, have any patient outcome? Now is it relevant? One way that we've done that, and this is, again, only one methodology that could be applied, is to really link donor product and recipient data sets. So we work closely with Nancy Heddle's group in McMaster, and Dean Ferguson's group in Ottawa where we can actually link donor information, so everything from the age and sex, frequency of donation, interval of donation, pre- donation hemoglobin levels, that we collect at the Blood System with the hospital transfusion service where they actually have recipient outcome data. So they have demographics. They have clinical characteristics. They have procedures. They have lab values. This is very easy for us to do in Canada because we have a national health system where these datasets are all linked together and we can actually do this quite easily. It may not be the same in other jurisdictions, but we take advantage of that in Canada. And we had two studies that we asked really two very different

questions. One was, does exposure to female blood, because of the perceived stability of female blood during storage, affect in-hospital mortality? And does manufacturing method affect in-hospital mortality, so looking at whole blood filtration or red cell filtration. Two methods of producing a red cell product in Canada that in the end have the absolute same ISBT code and label applied to them, because they're leukoreduced SAG-M red cells. We don't differentiate, so the hospital has no idea what they're getting. But working with us in the blood system, we can tell the transfusion service what they've received.

So when we do that, and I'll just give snapshots of the two datas, both of them have recently been published. The first we're looking at processing method. This is the work we did with Nancy Heddle. And I'll just highlight that. You know, we looked at 91,000 red cell transfusions into 23,000 patients over a period of time. And what we found when we tried to correct for as much of the bias as we can, and again,

respecting that this is observational studies, we can't show correlate causation. We can only show association. But what we saw was that there was an association with transfusion of fresh red cells produced using the whole blood method and in-hospital mortality relative to other treatment groups. So this was, again, surprising. It's telling us that almost opposite of what we've been trying to argue with the age of blood and the storage studies is that old blood is worse, fresh blood is bad. Well this is telling us that there is a characteristic of fresh stored whole blood filtered product that is associated with a poor transfusion outcome.

We did a similar type of study with Dean Ferguson's group in Ottawa where we linked 180 almost 190,000 red cell transfusions into 30,000 patients and basically showed that, you know, blood from young donors, 17 to 30, there was an increased risk of mortality in that patient group. Again, an association, not a causation, and that the increase -- interestingly there was risk from

female donors was even more significant. So for every single unit of red cells that a patient receives from a female donor, there's a 6% higher increase in mortality. Again, shocking, but the question is why? And, you know, personally I don't believe that it's actually due to age and gender of the blood. I don't think it has anything to do with females versus males. I think more importantly it has something to do about the characteristics of those red cells.

So what can we do to understand those characteristics? So just to sort of go back with the theme of what the session is about, you know, what can we actually show from intro vitro studies in terms of predicting outcomes? Well, I think one of the challenges we have is, and I think we've heard over a number of speakers now, is that our approach is probably flawed. We try to correlate in-vitro individual in-vitro parameters with radio labeling. And there's been a number of studies to do that. Dern was one of the early ones looking at ATP. Our group has done something

similar working with Larry Dumont's group and Sam Coker, looking at deformability and membrane changes, and you can show that these single parameters might be predictive of in- vitro labeling, but they're not strongly predictive. So it's going to be difficult for us to find that one biomarker, that one measure, that really is going to correlate with radio labeling.

But maybe radio labeling isn't what we want to be correlating with, and hopefully that's where the conversation's going to go in the panel. Now how can a

(inaudible) radio label survive one health patients, possibly predict what happens in complicated transfusion recipient communities that we're transfusing? So I think it's, you know, again, probably a statement of what we're trying to do and more so than what we're actually achieving. I think personally we need to do new

methods. We need new strategies to actually look at product characteristics. We really need to account for the natural variability that exists in the system, across the system.

So when I look at blood product quality, I really look at it from a variety of different lenses. One is what influences the donor having, and what can we do from a donor screening perspective, what can we do from a donor management perspective, to influence what's in the bag that ultimately may have an impact with the recipient. All of these factors, donor, manufacturing, storage, and the recipient, all interrelate in order to actually product the characteristics that we're trying to achieve. And perhaps, you know, we can even go as far as to say that we are entering the world where we have information now that we can actually design the right product using the right manufacturing method under the right conditions for the right patient.

So how do we do that more precision type transfusion medicine? So I think it's an interesting time that we're in right now. So thank you.

DR. SPINELLA: Now we're at the discussion panel part of the agenda, so if the speakers from this morning can all come up to the stage, to the table, we'll start that. So while I know everybody thinks they have a very loud voice, and most of you, you know, probably do, we do need you to come to the microphone to ask your questions. The FDA is recording the sessions today and tomorrow to help us with developing a manuscript eventually, but -- so please come to the mic. But we'll go ahead and start with the --

SPEAKER: Okay.

DR. SPINELLA: -- first question there.

DR. SWARTZ: So my name is Harold Swartz from Dartmouth. I'm not in this field so I have a very naïve question. A number of people have mentioned measuring the oxygen level in tissues, and my question is, how are you doing it?

DR. DOCTOR: I'll take a stab at that, thank you. That question, so there's not a simple answer. So one way that it's been measured is simply to measure oxygen consumption by indirect calorimetry. And, as you can see from some of the data I showed, that oxygen consumption really doesn't show us the relationship between delivery and consumption. So measuring tissue oxygen saturation is another way we get a little bit closer to it with new infrared spectroscopy or other indirect measures. This is pretty imprecise. It's an integrated measure of arteriovenous and tissue saturation. It doesn't work as well.

The dynamic assay that I was missing a slide for unfortunately, (inaudible) where you occlude a blood vessel, watch the rate at which the tissue desaturates, and watch the rate at which it recovers is perhaps a little bit better. But, quite honestly, we don't have a good way to measure oxygen delivery. There may be indirect ways of actually looking at mitochondrial

respiration by looking at cytochrome redox state through non-evasive. And that, in fact, may be the way to go. I was actually looking at oxygen utilization in the mitochondrial level during or before transfusion.

DR. KLEIN: But you're quite right, that is the question. Whether you're in the field or not, that's the question. And unfortunately or fortunately, human beings have so many compensatory mechanisms that the question is, where do you measure, and what is it that you measure that's going to correlate with clinical outcomes, because in the final analysis, it's the clinical outcome that matters and not the oxygen level. So the people who are measuring oxygen in the thenar eminence, which is easy to do, who cares what it is in the thumb, if it's the brain, the heart, or the kidney that are really at risk. So that is an important and very difficult question to get at.

DR. SPINELLA: All right, but Harvey, wouldn't you agree that that's where the use of

animal models would come in to help control many of those other factors and where you could measure oxygen delivery and consumption in specific tissue beds?

DR. KLEIN: Phil, unfortunately, I can't hear your question very well down here. The acoustics are bad.

DR. SPINELLA: It's the mic. Oh, it's the mic. I guess I was asking or commenting that, while you're right, in humans it would be very difficult to (inaudible) delivery. I think that's for animal models can come in and fill that need to a degree, where you can measure optimal delivery and consumption within specific tissue beds.

DR. KLEIN: I think there's no question, you can do that, and I think that we'll hear later is one of the values of having animal models. But, again, they have to correlate, sort of, with what we think are going to be the outcomes both in the animals and eventually in the humans.

DR. SPINELLA: Okay. Was there a

follow-up to that question?

DR. SWARTZ: I was just going to say, that -- it was not an entirely innocent question and so for those of you that are around, I think there are ways to directly measure oxygen in tissues, and I think it's a much needed addition in order to evaluate. I won't tell you everything, because outcomes are actually what really matters, but there are better ways than you've been using.

DR. SPINELLA: Okay. Well, I think during the animal session we're going to hear presentations that will hopefully link the (inaudible) delivery measures with outcomes. So hopefully we'll get some answers to those important questions later on. Another question?

DR. RAIFE: Thanks, Phil. I'm Tom Raife from the University of Wisconsin. So far today we have heard a couple of themes. One is that the biochemical qualities of red blood cells has market variability. And secondly, that the means by which we measure their efficacy are -- there's

a lot of doubt that's been cast on the accurateness of those assays. So, my questions is, going forward from here, would we propose to both change the means by which we measure the efficacy of red blood cell transfusions while we're also working on changing or standardizing the biochemistry of red blood cells, or should we go after one problem and then sequentially the other and, if so, in what order?

DR. SPINELLA: Jaro, why don't you try to tackle that one since it's kind of directed at --

DR. VOSTAL: I'm sorry, but I had a difficult time hearing that question.

SPEAKER: Microphone.

DR. VOSTAL: Turn the mic on, please. I'm sorry, I had a difficult time hearing the question, so you were asking about standardization of the biochemical tests? Nancy?

DR. SPINELLA: Between the echo -- Jaro, who's working with the audio here? Can we maybe try to get some -- I don't think it's hard to --

up here to hear what's being --

DR. DOCTOR: I heard the question, so --

DR. SPINELLA: -- said down there, maybe (inaudible).

DR. DOCTOR: Let me try repeating it. I think there's a funny echo up here that is making a reverberation. The question was -- actually, everyone in the audience probably heard it, but for you guys, so the -- should we prioritize improvement in the clinical trial outcomes or should we prioritize the quality evaluation of the pre- clinical product, and if those two are both out of phase at the same time, how do we know where we stand? And, in fact, you're right, that's where we are. And I think we're forced to try to do both at the same time. Right now there's several clinical trials that are trigger trials. I tried to make a case that, frankly, the decision on how to transfuse really shouldn't be based on hemoglobin concentration, and so we may not learn everything we should from that, but if we're wise about how we do the analysis, we may

still be able to suss out efficacy risk issues as a function of oxygen delivery. And at the same time, it's probably reasonable to consider functional testing of the blood product with what we think are the important parameters. I think oxygen delivery is probably important, so not just circulating. Do the red cells circulate? How do they influence oxygen delivery? But even more importantly, how do they influence blood flow? Because if they're impairing flow, even if they could deliver oxygen, you know, the oxygen isn't going to get where it should go. I don't know if that addresses your question, but you're right, it's a bit of a dilemma. We've got loose data at both ends of the spectrum.

SPEAKER: (Inaudible 0:43:11.)

SPEAKER: Have we got (inaudible).

DR. RAIFE: My concern is that on a big scale, if we have a moving target in terms of how we measure efficacy and a moving target in terms of improving the in-vitro quality of red blood cells, then I think it's hard to know where we're

navigating. An so would you standardize biochemistry and then with that major efficacy, or vice verse [sic]? So --

DR. ACKER: Yeah, you know, the point I wanted to make with my presentation was, one of the challenges we have is that we measure a variety of things pre-clinical for the evaluation, and then when we actually release that product into the manufacturing world, we get the compounding effect of donor variability in the manufacturing environment. And sometimes we often forget about how one decision in the transfusion medicine community can really have an effect downstream in the process.

So I'll give you the example. So right now there's a lot of concern over donor hemoglobin, you know, that we're iron depleting these donors, that these -- that our transfusion or donor hemoglobin triggers are not correct for certain patient groups, particularly young female donors, or young donors, so we're looking at raising those transfusion or those donor

collection hemoglobin levels, or the deferral period in order to actually make it safer for those donors. And that's the right thing to do. But what we forget though is that now we have a different input into our manufacturing process. So those products are actually going to be different. And we've actually started to see those differences as we've made some changes to our donor screening criteria in Canada where you see different populations of red cells now in those young donors, suggesting that, perhaps, iron depletion or anemic red cells in those young female donors might have been responsible for the effects that we were seeing in some of our data analysis studies.

So when you start to make changes in screening or you're implementing a new manufacturing method or a new piece of equipment, and even on the equipment side, you know, it's very difficult for the blood manufacturers or the makers of the blood bags to really understand how their blood bag's going to interface with someone

else's extractor, with someone else's centrifuge to produce a product that has certain characteristics. And we've got amazing data sets which actually show just subtle changes in everything from centrifugation to pressures on extractors can really change that characteristic. But those things aren't evaluated when they evaluate the blood bag. You know?

So how do we take account for all of these variables in the system when we start to look at transfusion outcome. And, unfortunately, the clinicians don't know when the blood system's made a change to their product. It comes with the same label. They don't know. So how do we communicate that better? So I think some of the studies that are being proposed, where you actually measure the product and then measure the outcome are going to be absolutely essential. You know? You can't make assumptions about the product that are going in and assuming over these two or three or four year RCT studies that that product hasn't changed. It likely has changed.

So how do you account for that?

DR. KLEIN: And I think the key on the transfusion studies is to have appropriate controls, which I would argue we haven't had until this time, so that, even if you have a terrible product, both arms get it. And if the product gets better or even changes during the course of the trial, ideally both arms would get the changes, and if the numbers are large enough, it ought to cancel out by randomization. But I think you're quite right. I think we really do need to work on both of those. They're different but related issues.

DR. SPINELLA: Next question?

DR. RAIFE: Yeah, I'll start out with a comment. I believe that blood flow is just as important as the ability to circulate for a period of time, and so the question is, are there data, or even reasons, to suspect that the different types of red cells made by different instruments or different ages might be different in terms of their ability to promote or not to promote blood

flow in recipients.

DR. DOCTOR: I'll take a swing at that. So, you had me at hello with blood flow. I agree with you entirely that blood flow is the principal determinant for delivery, so much more so than hemoglobin concentration or even concentration plus saturation in that both the rheology of the product, the, sort of, the pre-infusion rheology of the recipient, the vascular conductance of the recipient, and the dynamic interaction between both informs what will happen after the transfusion. And issues like the free hemoglobin or microparticles can change caliber, and as well as just sort of the simple biophysical properties of the blood. So the adverse impact of the stored red cells upon that physiology can't exceed the benefit from simply improving content. So you really have to be pretty anemic or you have to pretty volume depleted before the transfusion will provide benefit.

The other thing is, if we have to monitor this as an output when we're titrating the

blood and we don't really have good ways to monitor flow in humans other than feeling temperature, which seems pretty crude, measuring toe temperature or something like that. Or, as we'll hear later, perhaps, functional capillary density or some other novel ways to try to evaluate that in humans. So I think you've hit the nail on the head. It's key parameter and we don't use it right now, unfortunately.

DR. SPINELLA: Yep, and correct me if I'm wrong, Simone, but I think in the -- from the recess trial there was an ancillary study that attempted to evaluate flow in multiple ways and I think -- did that study finish, Simone, do you know? Or were they not able to --

DR. SPINELLA: So there will be data coming out. They had difficulties during the study performing these analyses for multiple reasons, but they will be there to -- being published soon, it sounds like, according to Simone.

SPEAKER: (Inaudible 0:50:05.)

DR. SPINELLA: I can't hear you.

DR. RAIFE: Okay. So that's the kind of information that needs to be published, the methods for measuring blood flow and differences that might be observed with different red cell products so that we could have some sort of a basis for evaluating the current and future products.

DR. SPINELLA: Yeah, I think they were using dark field microscopy as well as dynamic (inaudible) as measures within this ancillary study. Simone's shaking her head, so hopefully soon we'll get to see that data. Thank you. Mike?

DR. BOSCH: Mike? Mike, which one, comment then one question. Comment with respect to things changing during studies. We were, for a decade or more studying transfusion microchimerism, particularly in transfused trauma patients in collaborations with UC Davis in the latter years of that work, and we done randomized trials of leukoreduced versus nonleukoreduced or

tapped into those trials and seen no reduction in the rates of chimerism following early leukoreduction, but during the course of the ongoing studies, we saw dramatic reduction in the rate of observed microchimerism and that correlated with the change in the filter to a more efficacious filter. So they'd finally dropped below the levels of residual white cells that were needed to induce the chimerism.

And I had a question, although clearly tissue delivery is the key, survival studies are still clearly important, and two sort of question on that. One, I'd heard, and Larry's here next to me, that in a lot of these autologous survival studies that the healthy subjects were, kind of over time, selected for people who were giving better survivals and it was kind of -- these were cooperative people and, for whatever reason, (inaudible) --

SPEAKER: But wouldn't you do that, Mike, if you were a company trying to license something? Would you get the poor stores to do

your study?

DR. BOSCH: Yeah, so I'm just curious if that's sort of what John and Larry, whether there was validity to that assertion. And then the other is, what's been the progress in developing non-radio labeled survival techniques? I know there was work on biotin labeling and methods that could be used with much more, you know, confidence in terms of safety and potentially even in the context of real patients where you'd just take an aliquot and (inaudible) and look at survival in the context of real transfusions, are we making progress in developing a non-radio labeled survival technology?

DR. SPINELLA: Okay, John?

DR. HESS: I'm sure the repeatability of individuals in multiple studies varies from site to site. Certainly when I ran the site for the U.S. Army, I used myself in almost every trial I was involved in. I've done seven such studies on myself and so did my lab director. And we were consistently about 10 points apart. You know,

then that provided for us some internal validity. Almost everybody else in the study were young soldiers, you know, who were constantly moving through the institution. And so the data is essentially random. And when you go out and insist that you collect data on not just 10, but, you know, 50 people, everybody is scrambling for new donors. That's one of the advantages of increasingly asking for larger and larger size.

The second question, I'm sorry, I'm forgetting it.

DR. BOSCH: The labeling.

DR. HESS: Oh, the labeling. Actually, Tom is leading a project, you know, with looking at non-radioactive chrome labeling, Chrome 52, which has the advantage that it doesn't radioactively decay, so you can measure at multiple times. You know? And, you know, adding successive amounts, the current generation of induction coupled plasma mass spectroscopy allows one to do this with about five times the accuracy of current radio label study, just because you're

not exposing the person to 250 milligrams of radiation.

You know, the labeling with biotin has the potential problem of immunization.

DR. DOCTOR: And I want to answer that, as well. Actually, I want to ask a question since -- and it's for anyone who may know, particularly John. So my understanding is, so the clearance phase are all done in healthy people. The blood we give, everybody's ill. And, in fact, many of infections, many have conditions which influence the physiology associated with red cell clearance and survival. Even if we only consider survivalism as an important metric for storage, should we consider an expectation that survival in somebody with disease is actually a better metric than in a healthy volunteer? So the things that prolong circulation in someone with, say, sepsis may be different than the things that prolong circulation in a healthy human.

DR. HESS: I think the studies are done the way they are done because, you know, it is

socially acceptable to get a volunteer to donate a unit of blood, and accept his own blood back. You know? At this point, transfusing from one patient to another, at a time when we really don't know what the infectious and immunologic consequences are, most IRBs simply wouldn't allow us to do it. It's also a situation in which we assume is relatively free of immunologic consequences. You know? Getting your own blood back should have fewer immunologic consequences than getting anyone else's.

You know, what we're trying to test is the storage system. We're not trying to validate the model of transfusion. We're just trying to say, does this bag or this set of chemicals store the product in a reasonable way? And, you know, that's really what's -- I think safety concerns and practicality concerns really drive that. And, you know, the medical issues are separate.

DR. SPINELLA: All right. We're going to try to stay on time, so one last question, or last -- Jaro, I'm sorry. Jaro, did you want to

say something?

DR. VOSTAL: I just wanted to address Mike's point about alternate labeling of red cells. From a regulatory viewpoint we would be willing to accept these alternate methods. The only thing needed to be done would be to validate against a gold standard, which is still a chromium 51.

DR. SPINELLA: Andy?

DR. DUNHAM: Yes, Andy Dunham from New Health Sciences in Cambridge. I just wanted to make the comment, you know, as clinicians or manufacturers like we hope to be, all hope that these products work well, and I think that the real challenge here that -- from today, is that we continue to see this individual pieces of science that are adding up to different stories. And I think the challenge I give the folks here working on this is how do we integrate all of these different quality parameters, and then in the context of the heterogeneity of the donors to the work that Jason's presented, and then the

heterogeneity of the recipient, I think that the noise, the variability here, is as much a quality parameter as there is on these individual parameters that we're talking about. So I just wanted to make that comment.

I have a quick, naïve question, and forgive me if it's really silly. It just struck me today, how confident are we that the chrome, chromium 51, for example, sticks consistently within recipients? Because I can look at the data and can interpret it to mean that he chrome falls off of red cells differently in different recipients, so just curious about that assumption.

DR. HESS: We know that on average it leaves at about 1% per day, and as you say, it appears to be different in different people. Again, you know, large numbers help to average that, and we use that 1% fudge factor in determining the survivals. The actual recoveries, you know, that 75% number, is done without corrections.

DR. KLEIN: There are some data on that,

and there's very little variability in terms of the chromium eluting from different patients or donors' cells differently. There is some difference, but it's not -- over this short period of time it's not significant.

I want to make one last comment, if I might, because I heard my distinguished colleague here saying that the clinical trials showed no evidence that blood that was stored for long periods of time was --

SPEAKER: (Inaudible 1:00:01.)

DR. KLEIN: Maybe use another mic. It's an important point that if you actually read the conclusions of all of these studies (inaudible) what they say is quite accurate with the data that they have, and that is that, fresh blood is not (inaudible) average age of the blood transfused is about 22 days. No one has looked at blood in the last week of storage, and it'd probably be unethical to do that kind of study, except in animals. But as you saw from Dr. (inaudible) final week of storage. And there's certainly

animal evidence that this is toxic. So I'd be careful about saying that

(inaudible) day old blood is not superior to the standard practice in the United States and Canada.

DR. SPINELLA: All right, well, thank you, Dr. Klein. So we are going to go ahead and move on to the next session. Thank you to the discussants on the panel. And, Dr. Raife, you're moderating this next session, so you will introduce the next speakers.

DR. RAIFE: All right, our next, excuse me, our next session -- I can hear myself so I know it's working. The next session is The Methods for Detection of Red Blood Cell Processing and Storage Lesions, and our first speaker is Dr. Angelo D'Alessandro. Dr. D'Alessandro did his graduate work at Tuscia University in Italy where he focused his PhD work on red blood cell storage lesion. He's now an assistant professor at the University of Colorado Denver and Metabolomics core director. And Dr. D'Alessandro is going to

talk to us about omics of RBC storage lesion.

DR. D'ALESSANDRO: Well, first of all, thank you for giving me the opportunity to speak here today. It's pleasure in finding first. So I think it is rather clear by now that red blood cells storage in the blood bank results in the accumulation of a serious biochemical morphological lesion to erythrocytes. And most of them have been described by the previous speakers. I will not have a chance due to time constraints to get into the details of all these many complex regulations. What my group has done, of course, we are not the first ones to have studied blood cells lesion, and we'll not be the last group to study the lesion. What we bring to the table is the application of all mixed technologies, in particular, metabolics and proteomics. For those of you who are not familiar with these technologies, is the as comprehensive as possible study of protein as more molecule metabolize in a given system, in this case the red blood cells and (inaudible) and store it in the blood banks.

In the previous presentations, for example, in Dr. Glynn's presentation, we discussed about what are the next goal in the field of transfusion medicine. And some of the key questions that the field is asking to advance, the status of transfusion medicine in the next few years is to try and answer -- to try and give tentative answers to a few key questions such as, for example, what's in the bag, and how can we make better products? I think, and I hope that at the end of this presentation we'll be a little bit more convinced about this, that omics technologies can be used at least to describe what's in the bag. I think that to transform and make these observational studies even more relevant to the field we will need to be able to analyze thousands of units that technologies now from different donors, the technology is now there and available to analyze tens of thousands of units in a given -- in less than one year. So I think that, in the future, these omics observational approaches will become even more significant.

At the same time, I also do think that if we find a consensus on what a good transfusion outcome is, and for example, blood flow will be a meaningful one, I totally agree with Dr. Doctor. I think that omics technologies and the correlation of omics measurements to those transfusion outcomes will be irrelevant to advance the field by designing novel storage strategies or solution to make better quality products.

So I want to approach the beginning start with a Metabolomics approaches. These are just one of the studies we performed. We analyzed red blood cells in different solution, AS1, AS3, AS5, AS7 segment and we use this approach to try and understand what's in a unit of red cells, including both the SAG component and the supernatant fraction. We used to collect these red cells and supernatants on a weekly basis until the end of the storage period before analysis by we'll try performance (inaudible) chromatography and mass spectrometry. What we do find is that there is a series of changes in the small molecule

metabolized composition of red blood cells and supernatants on the X-axis here. This laser doesn't really work that well. On the X-axis here you have the (inaudible) on the Y-axis you have the different time points from blue to red is depiction of the relevant quantity of the given metabolite storage progress. And you can see that as storage progresses from the early time points until the end of the storage period, you have the progressive accumulation of (inaudible) metabolites and a progressive depletion of other metabolites (inaudible) blood cells to such an extent that it can actually draw a line that shows how these changes, not just accumulate but accumulate to a significant extent in (inaudible) red cells and supernatants.

You can do all sorts of (inaudible) analysis

(inaudible) that we perform as presented by Dr. Zimring earlier this morning to suggest that at least some of these metabolic

lesions significantly accumulating between storage date 14 and 21. And in most cases, 7 and 14 depending on the additive solution. Simplifying the concept, what we do observe is that, from an energy metabolism standpoint, these red blood cells tend to consume glucose and generate lactate almost in every additive solution you test. Consumer energy (inaudible) compounds in particular ATP and the (inaudible) which we know is relevant because this affects the (inaudible) binding core of hemoglobin and provokes a left or shift in the oxygen binding core of hemoglobin, therefore, promoting increases in oxygen separation.

Now, by increasing oxygen separation we have more oxygen that is available to promote

(inaudible) revised reaction to generate reactive oxygen species. And literally we have pretty much the same identical (inaudible) for reactive oxygen species generation in (inaudible) red cells, as storage progresses in the blood bank in this (inaudible). This chronology of evidence doesn't necessarily imply mechanism, but if a mechanism is there, then these reactive oxygen species, in theory, should be able to target the (inaudible) protein in the (inaudible) membrane of red blood cells and alleviates as more molecule and metabolites. Indeed, we do observe through which targeted (inaudible) approaches that hemoglobin, for example, in this case, I'm showing hemoglobin even better, is attacked by these reactive oxygen species accumulating progressively. A series of reversible at first, in the first three weeks of storage,

and progressively reversible, acidity of lesion to key function or residues such as, for example, cysteine (inaudible) 23 of hemoglobin better. And (inaudible) 92 and cysteine 94 if you count any (inaudible) of the hemoglobin better. And we know that these (inaudible) are relevant in mitigating (inaudible) the oxygen binding core of hemoglobin.

It may be argued that red blood cells are well- equipped with anti-oxidant system. There is more molecule level. For example, we know that (inaudible) extremely important in red blood cells to (inaudible) stress. And red blood cells are loaded with (inaudible) concentration of (inaudible). But, as storage progresses, these are the levels of (inaudible) are consumed progressively in red blood cells, and if you perform (inaudible)

analysis by providing available substrate in this very case glutamine, we can observe that very little of the glutamine that is provided exogenously actually ends up accumulating in (inaudible) and the majority of it actually is consumed to generate oxoproline due to a metabolic bottleneck in material blood cells which is caused by the absence of oxoprolinase, an enzyme that is involved in the recycling of oxidized (inaudible) cycle of (inaudible) back to (inaudible).

So if this hypothesis is correct, if energy metabolism to some extent correlated with antioxidant metabolism, then there may be some metabolic enzyme that has (inaudible) sensitivity functional residues such as in this case (inaudible), an enzyme that

converts (inaudible) to 13 (inaudible) substrate for the generation of 2,3-DPG. And these enzymes, for example, the relevant energy metabolism as I just mentioned, have the old sensitive (inaudible) sensitive (inaudible) residues assisting 152 and other (inaudible) sensitive amino acid residue such as (inaudible) 179. But in theory, if (inaudible) stress increases in stored red blood cells should be exposed to these oxidated lesion and affect the activity of these enzyme.

Indeed, this is the simplification of the model. If hemoglobin oxygen acceleration increases and oxidative stress increases, then there may be a mechanism where a red blood cell to oxidize (inaudible) to reduce, on the one hand, the decrease the energy metabolism in stored red

blood cells while promoting feedback backwards to other antioxidant pathways such as (inaudible) path which is one of the major pathways generally introducing equivalence such as NADPH to counteract oxidated stress.

To test this hypothesis, first we perform switch

(inaudible) analysis to confirm that as storage progresses, (inaudible) is actually at first reversibly oxidized and later on irreversibly oxidized in both red blood cells cytosol and progressively migrating to the membrane and supernatants. And these corresponds to the (inaudible) activity of (inaudible) phosphate (inaudible), in particular the one (inaudible) in the supernatants of stored red blood cell is the fraction of (inaudible) that has the highest

loss of activity. If this were true, than these old mechanisms should correspond to an increased tentative activation of the (inaudible) pathway to generate (inaudible) to counteract oxidative stress. We tested these by incubating cells with heavy liberal substrates and performing increasing experiments without entering into much detail. We can now determine (inaudible) plus two divided plus three to determine (inaudible) and if this ration increases, as we did observe, and it did increase from a -- to a significant extent starting from storage day 21, we can tell that at least the red blood cells tried to cope with oxidative stress as storage progresses.

However, there's additional mechanism

that red blood cells try and exploit to get rid of irreversibly oxidized (inaudible) and lipids. If you measure the absolute quantities of oxidized and (inaudible) supernatants, but also of oxidized (inaudible) and oxidized series of other proteins in red blood cells supernatants, we can now exploit quantitative absolute quantitative (inaudible) approaches to determine the absolute levels of these oxidized proteins in the red blood cell supernatants, and use that as potential mile markers not just of the energetic lesion, but also as the (inaudible) stress lesion of (inaudible) red blood cells. And again, I don't know the relevance of any of these metabolic parameters, or

(inaudible) parameters, but I think that understanding this mechanism may help understand, for example, why these red blood cells using these sort of

(inaudible) mechanism try to get rid of membrane portion through the form of (inaudible) to remove irreversibly oxidized protein lipids through a form of circulation. Unfortunately, as it has been mentioned before, removal of membrane portion through recirculation results in the progressive decrease in the surface to volume ratio, which makes these red blood cells more susceptible to hemolysis and to hemolysis-induced by, for example, mechanical fragility or osmotic fragility as I'm showing here.

So the whole point I made in this first part of the presentation, I just have a few

additional slides left, is that if you understand the mechanism that make red blood cell store the -- that promote this sort of lesion of these red blood cells, we can try and come up with (inaudible) strategies and solution to counteract this lesion. One of the approaches that we try and investigate in our lab in collaboration with (inaudible) scientist and before coming here to the States with Italian Nation of Blood Center and (inaudible) is an aerobic storage of red blood cells. The rationale behind the removal of oxygen, more than an aerobic storage should say, (inaudible) storage of red blood cell, is that by removing oxygen, you promote, at first, alkalization of the intercellular compartment of red blood cells. I don't have the time to enter through the details of the promotion of the (inaudible) fact, the removal of (inaudible) and the

(inaudible) equilibrium that promotes the fusion intracellularly of (inaudible). But all these mechanism contribute to the alkalization of (inaudible) upon removal of oxygen or the decreasing the oxygen acceleration. And these affects, positively affects, the activity of key enzymes (inaudible) pathway, promoting both energy and antioxidant metabolism. This same approach, this same beneficial effects, can be achieved, for example, in high bicarbonate (inaudible) loaded additive solution, which I will not have a chance to discuss today, but we can use omics technologies to investigate how these and evolution of the solution can actually help improving the red blood cells storage lesion, mitigating the red

blood cells storage lesion.

The additional rationale behind the effectiveness of the removal of oxygen is the promotion (inaudible) called oxygen (inaudible) metabolic modulation by promoting the oxygenation of hemoglobin. You promote the oxygen hemoglobin binding to (inaudible) three, which promotes the localization of GAPDH and other key rate limiting enzymes of (inaudible) and since when they're bound to (inaudible) three and their high oxygen separation condition, these enzymes are less active. The localization in the (inaudible) make them more active by and thus fuels the energy metabolism in stored red cells.

And finally, the probably over simplistic rationale is that by removing oxygen you're going to move a key substrate to promote the reactive oxygens species generation, therefore mitigating the oxidative stress lesion and therefore reducing the necessity to recirculate the irreversibly oxidized protein cell lipids. At the same time preventing the necessity to induce

that GAPDH oxygen (inaudible) stress dependent modulation that I mentioned in the first part of the presentation.

So here I'm showing, for example, a study that we just published on blood where we have (inaudible) blood cells will progressively increase the oxygen acceleration of storage progresses in the blood bank. We have the hyperoxic red blood cell here in violet where the oxygen separation is maintained constant 25% or higher throughout all duration of the storage period, and then we have the deoxygenated red blood cell control test, hypothesis, where deoxygenation is around 5% oxygen separation throughout the whole storage period. And as a result, we did observe that in the hyperoxic red blood cells, levels of oxydated stress as a measure, for example, spectrophotomatic (inaudible) through (inaudible) measurements, but also through targeted absolute (inaudible) per the omics, and which targeted (inaudible) per the omics are decreased in the hyperoxic red blood

cells and increase in the hyperoxic red blood cells.

Consistently, DPG preservation and ATP preservation were higher at least until storage day 21 for DPG and throughout the whole storage period for ATP in the epoxic red blood cells in comparison to the hyperoxic red blood cell. And then (inaudible) control. And these corresponded to a decreased activation of the (inaudible) pathway and the epoxic red blood cells despite activation still being there, suggesting either a decreased oxygen dependent metabolic modulation due to the (inaudible) three oxygen dependent model, but also at the same time, probably the decreased necessity of these hypoxic red blood cells to counteract oxalated stress. And, indeed, by measuring directly

(inaudible) levels and a whole other series of antioxidant there's more molecule metabolized enzymes, we did find that (inaudible) reduced to oxidize

(inaudible) were higher throughout the whole storage period in hypoxelated blood cells in comparison to control.

All of these translates into preservation of the morphologies of these red blood cells by the end of the storage period. I'm just here simplistically, highlighting the (inaudible) and spherocides in the 42 days old control versus the anaerobically (inaudible) red cells, which translates in a reduced hemolysis and a reduced osmotic fragility of these red blood cells anaerobically in comparison to controls.

Of course, this is just one of the strategies we can pursue now that are already in place to mitigate the storage lesion, whatever the clinical relevance of the storage lesion is. And again, I will be sending in for sure applications to try and correlate the observational status with functional outcomes with clinical (inaudible) that are in this audience. But at the same time, I think that, you know, I discussed about the

anaerobic storage of red blood cells. It has been mentioned something about a rejuvenation solution and alkaline additives such as AS7. There are already solutions and strategies and there may be even better strategies and solution that can come up in a very well-designed way, applying the results, the information we obtained from these observational omic studies to improve the quality of red blood cells storage. And I think that in the next few years we'll have a chance to further investigate this.

And thank you for your attention. I would like to thank all the equal operators to a different extent we that made this research possible. Thank you.

DR. RAIFE: Our next speaker is Dr. Bernhard Palsson who is a professor of bioengineering and professor of pediatrics at the University of California San Diego in La Hoya, and also a principal investigator of Systems Biology Research Group in the department of Bioengineering, and he's going to talk to us about

Systems Biology of a Red Blood Cell Storage Lesion.

DR. PALSSON: So I'd like to thank the organizers for inviting me to speak to you about their work. And I am especially thankful for them for putting me right after Angelo's talk, because that's perfect introduction into what I'm going to be talking about.

As you know we can now profile cells in molecular detail, extensively. And the analysis of all of the data has given a rise to a field that we call systems biology. And I'm going to try to describe to you today how those approaches, the approaches of systems biology, are being used now to analyze omics datasets coming from stored red blood cells. And, I guess this is it. Yes.

So here's the process. So as you know, in 2000, the first draft to the human genome sequence came out. It was called Build One. That draft became better and better, and by 2005 we had Build 35 that covered 99.99-some percent of the euchromatin, and at the time we were getting good

genome annotations associated with the human sequence. Okay, oh, this one. And at that time we undertook the effort to reconstruct the global human metabolic network which was, at the time, comprised of the function of 1500 genes that was published in P&S in 2007. The second build came out in Nature Biotech in 2013, accounted for about 1800 gene products. And the recon three is about to come out. It is based on 207,000 human gene products. And that's, interestingly, a pretty big fraction of the 19,000 annotated human genes, functionally annotated human genes in (inaudible).

So we can build that network. That's the global map that is encoded on the human genome. That can then be tailored to a particular cell type and for the red blood cell, deep proteomic datasets started coming out in the last 2000s. And in a few years we developed something like 30 of them in the literature, and you can take all those peptide fragments and map them onto these reconstructions and pick out all the metabolic genes products, all the metabolic

enzymes that have been detected in the red cell. So I'll show you that in a moment, but that is a metabolic map for the red cell based on the human genome sequence, as well as the proteomic datasets.

Then we can look at the state of that metabolic network by getting a time series of data, as I'll show you in a moment. This is relatively new set of methods and in the bioengineering department at UCSD we have a graduate class on systems biology for which this book was written, and every single lecture that has been recorded and is on YouTube, in case you want to learn more about this methodology. These are not models, but they're based on enzyme kinetics and biophysical phenomena, but they are more network models of the source that traffic engineers, for instance, use to calculate traffic patterns in cities.

So I'm going to talk a little bit about how you use these reconstruction for analyzing omics datasets generated from red cells in cold

storage. So the first thing I'm going to show you is what the baseline metabolic decay looks like. So basically we sample, I think, about 15 (inaudible) points

(inaudible), about 15 times over the 42-day process, and we generate different data types. We get this so-called exometabolome, what's in the median, the antometabolome was inside the cell, and various other measurements like pH, PO2, and so forth, the routine blood bank measurements.

Angelo showed you, I guess, sampling every seven days or so, so this is a little finer time grid of data we have here. So this is what a data matrix looks like for every bag. There are 135 measurements being made at, let's see, I think it's about 14 data points if I remember correctly. Many of these measurements are in triplicate, so there are literally thousands of data points generated for every storage blood bag. I'm going

to show you some calculations from 10 donors, five male, five female. And they were age balanced.

The metabolic network that results from the process that I described to you earlier is shown here. It's compromised of 283 metabolic reactions. Many metabolic pathways had previously had not been discussed or described to be active in the red blood cell. This is lipid metabolism, quite a bit of new lipid metabolic pathways associated with lipid metabolism discovered here. This green box is the set of pathways you see in a typical hematology textbook. Okay, let me see here. Wrong button.

So here's the workflow that we use. We have a bunch of time dependent profile like this for metabolites. We do multi-variant statistical analysis first to look at the correlations and the patterns in the dataset. Then we actually get quantitative decay rates or build-up rates of metabolites. And that quantitative information can go into these network equation that I showed you before. And based on that you can estimate

the most likely metabolic flux map for the red cell under any time point for which you have this data.

I'm not going to have time to go into this. This is a little bit detailed and can't be described in 15 minutes. But I'll talk to you a little bit about the overall patterns that one can decipher from these datasets. So here's a snapshot of the data, as I guess Jim Zimring showed this morning. We and Angelo have seen this three-phase pattern in the dataset, where there are kinks at day 10 where metabolic state shifts and another one at day 17 where it shifts again. And since there has been some discussion about the last week of storage, after day 35, I should not that there is a subtle shift right around day 32 to 35 also in this dataset that hasn't been described much in the literature. Here are some individual decay profiles. Here is ATP. It goes up for the first 10 days, and then it decays. And here is the 35 data point that I talked to you about. Well known pattern for 2,3- DPG,

(inaudible) somehow starts to degrade and that second shift is most likely related to redox metabolism as Angelo described. For instance, here's (inaudible) being consumed. And here's that hypoxanthine that Jim Zimring mentioned this morning. This has always worried us a lot, because this is 0.4 millimolar and this is quite the high concentration. And I wouldn't be surprised if this actually becomes a biomarker of some utility in the future.

As SAM actually, the SAM metabolism, is active in red cells and SAM builds up as a metabolite during these first two phases of (inaudible) and SAM is involved in methylation. I don't know what is methylate being in the red cell. Maybe somebody else knows. But the ability of red cell metabolism to carry out methylation reactions decays after that second shift.

Here I am a little bit on thin ice because I didn't carry out this analysis, but the obvious question is, is there any correlation between these metabolic states you can measure and

red blood cells in their storage, and clinical outcomes. We don't have an answer to that question, clearly. But we were able to do a couple of analysis here that's worth mentioning and they're detailed in this paper.

We got access to the Danish registry of transfusion. And we started calculating relative (inaudible) ratios of mortality after seven days of blood transfusion, and the results are shown in this table here. And there is a clear kink in that calculation, and that odd ratio is around 10, which coincides with that day 10, which is the first shift. And the curve that I showed you, you also got eight volunteers to undergo autologous transfusions and they donated blood three separate times, stored for a week, two weeks, and three weeks. So in the middle of these three phases are then transfused, and the statistics are not a grade from just eight volunteered recipients, donors and recipients, but they then the paper shows that there is indication that (inaudible) damage markers are higher with the transfusions

from phase two or three compared to phase one. So not a conclusive answer here at all, but some interesting information.

Now, are there actionable biomarkers in this dataset? We got together all the datasets that we have, which are all (inaudible) and tried to find out the best extracellular measurements that would allow us to distinguish between these phases and the eight measurements that showed up, and they're shown here. Angelo also provided the data from AS3 and these same biomarkers apply there. This is now online and blood, I don't think the final publication is out yet. It was interesting to see from Dr. Zimring that hypoxanthine and xanthine were the only correlates he could find in his mouse data, but not the others, so that's why I'm stating that perhaps this will become useful biomarkers for quality of red cells in other storage conditions.

So a summary of some our findings are outlined here. So big data analysis of deep metabolic datasets reveals these three metabolic

states that red cells undergo during a cold storage. When you do some of the calculations you realize that 2,3-DPG may actually go through the mutase and get a reverse reaction, and the thermodynamics support this. And what's important about that, if this is true, is a proton is consumed there, and so the pH is buffered during phase one by that reaction. And, of course, when this is degraded to lactate, you get two ATPs, and ATP is building up during phase one, and then decays.

There are surprisingly high levels of malic acid found in the red cell. Some of them come from the citric buffers that are used during the preparation of the cells before they go into the bag, which were shown by C13 label citrate in that preparation. This is over a millimolar, so this is quite a high concentration. And the fact that citric acid can be converted to malic suggests that there are some remnants of the TCA cycle in red cells. So this was surprising.

Extracellular mannose and fructose that

come from the donor are sometimes at reasonable levels and they are consumed very rapidly and gone by day eight. They disappear during the first phase. And there is active SAM metabolism during phase one, as I described earlier. And we now have eight biomarkers if we want to distinguish between these three phases, metabolic phases in stored blood.

We looked at the extracellular ones and, of course, you would love to have a non-invasive measurement that could just look through the blood bag and measure that concentration if you wanted it.

So we have done quite a few (inaudible) from that state to see if we can change it. And I'll just show you some data along those lines. We have looked at the metabolic fate of adenine quite carefully, and dosed it, you know, as you saw it's depleted by the end of phase two. We've looked at storage temperature for -- as I'll show you in a moment, for certain reasons. We have looked at fructose and mannose since that was

observed as I described earlier, and we are now looking at spiking the media with the precursor for glutathione. So the (inaudible) pulse, this is either published in transfusion or just about to be. I think it's already available on the Web, so here's the pattern that I showed earlier. And we decided to spike adenine at the end of day 10. That is shown here. This is the extracellular adenine concentration. In the middle of this phase, day 14, then at the end of it, those panels are shown here.

And then when we analyze the data with this PCA plot that I showed you before, we see no difference between the pattern here, this three phase pattern with and without the spikes, and we also actually carried out an experiment where we just doubled the adenine concentration from the beginning. And this does not appear to change this pattern at all. In fact, when you double the adenine, at the end of the second phase, on day 17, there is just a residual amount of adenine left that is not consumed. So extracellular

adenine did not seem to change this pattern very much. It did influence SAM metabolism a little bit because adenine is a precursor for SAM, so this is a subtle effect, but it is there.

We looked at these alternate sugars, as I mentioned. We prepared bags with elevated amount of fructose and mannose, and the results here are of some interest. So fructose has a very negative effect on ATP levels. If these two sugars are in too high levels in the bag, the 2,3-ATP G concentration, the 2,3-DPG concentration, decays a little faster. And if you look at glucose, this is actually extracellular glucose, not cytoplasmic, you see it doesn't drop as fast when you have mannose in there. They use the same transporters and just compete for it. And all indications are that once inside the cell, glucose and mannose are degraded the same way, but fructose enters the cells somehow differently, and it has a very pronounced effect of the chloride concentration and also in sodium. But most of the metabolic processes are not that different between

mannose and the glucose, but fructose seems to be -- would be a bad additive. It is again, this PCA plot and the controls in the elevated sugar concentrations, and the basic pattern does not seem to change much by using these additives.

Okay, I think I mentioned these three points here. So glutathione precursors, so we have put the three amino acids in there, or alpha-Ketoglutarate as a proxy for glutamine. We put them and labeled it here, and we just don't have the full data analyzed yet, but it seems like we hit jackpot here. The pattern of decay, in terms of these three metabolic phases seem to be completely different when you add these precursors to the median. So maybe these could be considered as additives to a future storage median, but it's too early to make a statement in that regard.

Now, the last (inaudible) being made on the storage conditions that I want to talk about is temperature. If you do one of these experiments, it's painfully slow. You have to wait for 44 days and then you run the mass spec

and then you analyze, and before you know it, three months have gone by. So for those people that do omics datasets, like to do things at high throughputs, so we asked ourself, can we just speed this up by changing the temperature a little bit? So we did that experiment and that's shown here. So we picked 4, 13, 22, and 37. This is the storage for temperature for platelets. And we measured the decay rates of all these metabolites at these three temperatures, and so we get slopes, or the equivalent of the slopes or curves like that. Then we plot that slope as a functional temperature and we can get, then, what's called the Q10 value, which is the rate of change for every 10-degree change in the storage temperature. And this here's a histogram of Q10s for all the measurements we make, and the average here or the median, I think, is around two and a half. So if you were to go from four degrees to 14, the experiment could be done two and a half times faster if the temperature doesn't change the pattern of decay and here's some of this data

shown. You know, there's different concentrations at these different temperatures. Here is that three phase pattern that I've been showing at four degrees, and it seems to be preserved at 13 degrees, but it does change entire temperature. So potentially you could do these experiments to accelerate them at 13 or 14 degrees. We have not done any experiment at 14 degrees since we did this. So I don't know if this will ever be done in practice.

So this is actually the first time when there's a dataset available that measures temperature effects that precisely and that comprehensively in a metabolic network and the answer here is about two and a half, full change, for every 10 degrees. We could probably do experiments at 13 degrees like I mentioned, and still be looking at the same decay pattern, and you know, if we decided to do this experiment en masse at that temperature, we would be able to do things more quickly.

So here's a summary of my talk, so the

systems biology of red cell metabolism has advanced in recent years. We now have a number of deep coverage metabolic datasets for stored red cells under a variety of conditions. We see this three phase metabolic decay over and over again, reproducibly under multiple conditions. We now have good extra cellular biomarkers to measure them or detect them and distinguish between them if that's what we want to do. We are trying to (inaudible) that pattern as I showed you, to see if you could change it and if changed, would it potentially lead to better storage solutions. No definitive results there. We now have these system biology tools, these models for designing the next generation of storage solutions, so we are trying to do that. And I would like to state at the end that we really badly need a big data base for all of the data that is being generated

on red blood cells under storage conditions. We are in the era of big data analytics. There's a lot of very skilled people that do big data analytics and we certainly have a lot of data to put into these databases.

Now, I don't know if you've heard the term to be bricked, you know, if my phone is off the Web, or off WiFi, it's like a brick. It's bricked. It's useless. It could just as well be a brick in a wall, so that's the term that computer scientists use to describe disconnected devices. They are bricked. And the same thing is true for disconnected datasets. If you have a dataset on red cell decay under storage conditions and you can't contextualize against all the other data that's available, it's effectively bricked. It's sitting by itself in an Excel spreadsheet somewhere. So we really need to build up a big database for big data analytics for this field.

Finally, my acknowledgements, so James

Yurkovich is with us here today. He's done many of the analysis that I showed. Aarash Bordbar has spoken at meetings like this before. He really drove that foundational paper that I talked to so much about, about the three phases. He and I have cofounded a company called Sinopia Biosciences that's starting to look into these storage solutions. So that is a disclosure statement. This company has actually received SBIR grants from Seymour and Glynts program here and is trying to look at some additives to see if they changed that pattern and improve it. Pierre Johansen did the healthy volunteer study that I showed you in Denmark and also facilitated access to the data's registry that I showed you the results from. Most of the blood bank storage experiments were done in Reykjavik and the analysis was done at the university there. And Giuseppe Paglia generated all that data, obviously, of Italian descent and it's kind of an interesting historical accident that Angelo Giuseppe generated all the first big datasets, metabolic datasets for red blood cells

in the literature.

With that I'll stop and leave the podium. I guess there are no questions. Thank you very much for your attention.

DR. RAIFE: Thank you, so the next talk is me. I'm Tom Raife, Professor of Pathology and Laboratory Medicine at the University of Wisconsin now and Director of Transfusion Services there. My talk is on the genetics of red cell storage and studies of twins. These are some of my collaborators, John Hess, University of Washington, and my many collaborators at the University of Iowa, and geneticists here and biochemists, and then my newest collaborator is Josh Coon and his group, who do mass spectrometrics at UW.

Alright, so we've seen this chart once before. Dr. Klein showed this earlier and the key features is as Dr. Klein pointed out is that this study by Dern and Workowski was, I think, 28 different subject experimental, experimental subjects that they used for in vivo recovery

studies of red blood cells. They were working on red blood storage in the translational way of looking to improve blood storage and what vexed them about their work was that was so much variability in terms of in vivo recovery among theses 28 experimental subjects. But the key thing also here in the context of this publication is not only was there a lot of variability but within individuals there was tremendous consistency. So as has been said before many times here, an individual is very characteristically, they store poorly, or they recover poorly or they store better or recover better, and I would say just by a review of my bio that among the many issues that we discussed here that this is to keep things sort of simple, this issue of recoverability of red blood cells after storage have been really the focus of our work for the last six years and I would argue that among all the different ways we could improve red blood cells that improving the recovery seems like one that is laudable and as is hard to imagine how

donors who store like this when nearly half of their red blood cells are no longer in circulation the day after they are transfused, could somehow be better than donors like this. So, that has been the focus of our work.

This data also from Dern and Workowski, a follow on from what I just showed you, so they went looking for biomarkers of red blood cell storage so that they didn't have to do chromium 51 labeling on all of their subjects, and among about a dozen or so biomarkers that they looked at, ATP stood out. Now, we've had a discussion of how the value of post storage ATP as a biomarker of recovery and the data that is shown here, I think speak for themselves along with data that was shown from of Dr. Hess's studies. Dr. Ernie Beutler's comments notwithstanding, I think that ATP is still a reasonably good biomarker of in vivo survivability of red blood cells and so that is what we have really focused our work on.

Predicated on this notion Dern and Workowski, back in late 60's conducted a family

study. They surmised that the in vivo recovery that they were seeing was perhaps a genetic property of the donors and that ATP would be a good marker of that so they studied post storage ATP levels in red blood cells in, I think, 32 families, about 105 individuals or so, and they published a paper that concluded from the statistical analysis that over 95 percent of the variability in post storage ATP levels in these various subjects was heritable and because that statistical arguments is not very easy to show on the screen, I did my own analysis from their data tables by simple calculating the mean value post storage ATP from the parents in these families and correlating that with the mean value from all the off spring that were in each family and as you can see from this curve there is a strong correlation and that just convinced me that indeed their conclusion was valid.

Alright, so this was not the only work being done on ATP levels and heritability or the genetic determinants. Back at the time, George

Brewer at the University of Michigan was working on this issue as well. Here is a study that he published in the late 60's and concurrent with Dern and Workowski's work in which he compared the ATP levels in pre- storage red blood cells in two different racial groups, and as you can see there is a significant difference in these two racial groups in the pre-storage ATP levels. So this lent more credence to the idea that indeed ATP levels in red blood cells is a heritable trait.

In Dr. Brewer's lab there was a grad student, Tom Gilroy, who did his PHD thesis on the genetics of glycolysis and red blood cells, published in 1974, later published in this manuscript in 1979, and just to make this table very simple, so he did family studies and he looked at all the glycolytic intermediates from glucose-6 phosphate through pyruvate as well as the adne nucleotides here and calculated heritability taking into consideration a variety of variables that might impact on heritability and I made it simple by putting arrows next to the

metabolites that he found to be heritable. These are the example heritability estimates, so he found, for example, glucose 6 phosphate - fructose 6 phosphates to be essentially a 100 percent heritable in his family studies and you can see that then among all of these metabolites and the glycolytic pathway quite a few of them are represented as being heritable.

So, I would just summarize that historical data that by the late 1970's (inaudible) understood quite a lot about energy, metabolism and red blood cells and that there was a strong heritable component of energy, metabolism both I glycolysis and in the production of ATP. So that was a jumping off point for our studies. At the University of Iowa when I was there we did a twin study that with the aim really of reproducing the Dern and Workowski data on the post storage ATP levels along with some other objectives from my collaborators, so twin studies we wound up with 13 pairs of monozygotic twins and 5 pairs of dizygotic twins, these were confirmed

by zygosity testing and they were recruited and donated a standard autologous unit of blood were qualified by our autologous donation questionnaire and those blood units were stored and then sampled at various days afterward with the initial intent of measuring ATP along with glutathione pathway components, et cetera, and we added on to that later metabolomics scans and more recently proteomic scans to see what we could learn about heritability.

So just for those of you who are not familiar with twin studies conceptually what goes on there is that if there is a measurable trait in individuals then within monozygotic twin pairs the variability within the twin pair is smaller as a function of the total variability in monozygotic twins than compared to dizygotic twins and that's easily calculated using interclass correlation. So that's how that works.

A couple of things were published already from that study, data sets and some of you have seen this I'm sure, so we measured ATP in

both CPT2D storage and AS3 here at day 28 you can see our heritability estimates are in the 50 to 60 plus range and now when we measured the delta ADP from day zero through the end of storage, actually 56 days of storage, also a heritability estimate in the 60 to 70 range. So, basically from this part we concluded that we confirmed the data from Dern and Workowsky that indeed ADP levels post storage are heritable.

So, now I'm going back to this table that I showed you earlier from Tom Gilroy's work and having now conducted a metabolomics scan we were struck by the fact that within the glycolytic pathway our heritability calculation showed quite a cluster of a heritable metabolites as well. And, so, my looking at this data along with Gilroy's data I'm sort of a lumper, so having read his thesis I had a lot of faith in his heritability calculations and in ours as well, and so when we combined the measures of heritability that we found here and these would be anywhere from pyruvates about 62 percent heritability

estimate of up to, I think, 85 percent for DPG so significant heritability estimates we find that virtually every metabolite in the glycolytic pathway is heritable. I guess the only gap is gap itself in this particular data.

So, we have confirmed, we think, that the activity of the glycolytic pathway in pre-storage red blood cells is a heritable trait and biological variability in our data set is on the order of about 9 fold on average between the lowest and highest individual in our study. That's illustrated here.

The other key point, when we do correlation matrices on our data and the same with Gilroy's data, we find that the heritability of theses metabolites is not random, but rather that the majority of the metabolites within the glycolytic pathway are positively correlated and that suggests that the entire pathway's activity level is inherited on block so to speak. So I've illustrated that here by showing a pair of monozygotic twins that have higher levels of

glycolytic metabolites compared to a pair of monozygotic twins that have lower levels of glycolytic metabolites.

So, moving on to our more recent analysis, the proteomic analyses you can see the coverage we got from those and the metabolite analyses here, I'm just going to focus among these. This is heritability scores or estimates for a number of different proteins and then metabolites here as well. The one that stood out, and this took a while to recognize, but when we got around to looking at all these metabolites actually carbonic anhydrase CA1 specifically was the second most heritable, or had the second highest heritability estimate of all of the proteins that we scanned in red blood cells, 84 percent heritability estimate, and when we got around to doing correlation matrices of everything against everything here, carbonic anhydrase among all of the metabolites that we measured had the strongest correlation with end of storage ATP that was measured day 42. And, so since we were

looking for markers in fresh red blood cells that might somehow reflect end of storage ATP that became of interest to us, and so we began to suffice how that might be the case, you know, knowing the activity of carbonic anhydrase we thought that perhaps it was modulating PH and by the grace of God one of my collaborators had been measuring PH on at least some of or about a third to a half of our subjects had PH measured at various time points so we were able to go back and ask the question: could carbonic anhydrase be modulating PH? One more important point was that carbonic anhydrase correlated negatively with end of storage ATP, so the higher the inherited level of carbonic anhydrase the lower the end of storage ATP. So we thought that perhaps that was because carbonic anhydrase might be generating acid in these subjects and that the acid would be inhibiting phosphofructokinase and shutting down glycolysis. This is the scatter plot between carbonic anhydrase and ATP, albeit not the most exciting correlation in the world, but a level of

significance that convinced us that there is indeed some relationship there, and then here is carbonic plotted against the mean PH value and so what I noted was that when I plotted carbonic anhydrase concentration versus pre-storage red blood cell PH and then day 7 and day 14 there was a positive significant correlation for each of those days and then the correlation fell apart which is in keeping with what we've been seeing in the last several talks that there seems to be inflection point at about

weeks of storage where things change albeit a fairly weak correlation, but nevertheless it was a negative correlation so it suggests that the higher the inherited level of carbonic anhydrase the lower the PH in that subject's blood. The one last thing we were able to do then was correlate the mean value of the PH of these 3 days against day 42 ATP and as expected there is a positive correlation. So, the lower the PH in these individuals then the lower the end of storage ATP.

That gives rise to hypothetical models that we have developed here and so the notion of a low post storage ATP model. These four factors are all heritable these in the sort of 50 to 60 percent range of heritability estimates, and it turns out that carbonic anhydrase and this phosphoglucomutase are also fairly strongly positively correlated as those co- regulated at a transcriptional level, so we kind of developed this model where we say that if you inherit higher levels of carbonic anhydrase you generate more acid and that has potential to inhibit phosphofructokinase. If you happen to inherit higher concentrations of band 3 as we saw earlier in the presence of oxyhemoglobin that has a potential to sequester the enzymes in the glycolytic pathway also inhibiting glycolysis and then if you inherit higher level of this phosphoglycerate mutase that actually loses an opportunity to make an ATP at this step here, all of which give rise to what we suggest is a lowered day 42 ATP and then this is just vice-verse of

that.

So, I've concluded so far that red blood cells glycolysis is clearly heritable. The data from Tom Gilroy and our data are concordant and suggest that there is a significant heritability that (inaudible) what the genetic determinates are. We do not yet know, and we propose that inheritance of carbonic anhydrase concentration could be one factor that is important as a genetic determinate of red blood cell storage.

Thank you. (Applause) The next speaker is Dr. Michael Busch, he is co-

director of Blood Systems Institute and Vice President for Research and Scientific Affairs at Blood Systems in Scottsdale, Arizona and Professor of Laboratory Medicine at UC San Francisco, and he is going to talk about the RED-III omics studies.

DR. BUSCH: Thank you. It's a pleasure to be here, I appreciate being part of this session, it's really, I think, a natural flow here because I think as you'll see, the RED-III RBC

omics program really is trying to deliver the big data sample set and repository that it's been alluded to earlier.

So, the RED-III Program, just for broader context is a program that consists of four large blood centers and affiliated hospitals where we're tracking all the components transfused to these hospitals and all the clinical outcomes of patients in these hospitals, a Central Lab coordinating center at RTI, there are also international programs; Brazil, South Africa, China.

The program has instituted a large number of studies initially studies, some of them, infectious disease, allimunization, et cetera, and then larger prospective studies that we've executed in the phase II and I'll be focused on the RBC omics study. So, and this really was precipitated by some discussions with Mark Gladwin, I think in the context of some of the earlier NH funded research on the storage lesion and the argument that has been extensively

discussed by Tom and others earlier that there seems to be a consistent heritable component to the storage lesion and the capacity of red cells to tolerate extended storage and the hypothesis that there may be substantial differences between donors, not only attributable to age and gender, and we'll see data on that and that was reported earlier by Jason and Tamir, but also more extensive differences attributable to genetic ancestry, many of these potentially inherited as a consequence of malaria induced polymorphisms in various red blood cell parameters. In addition, there was interest led by Alan Nast at (inaudible) Wisconsin in terms of the principle of super donors. Some donors seem to be able to tolerate a very frequent blood donation and a sort of hypothesis that are these super donors either extreme hemolyzers or extremely better in terms of blood storage. So this merged into a program, although

these would seem to be somewhat disparate hypothesis and concepts and might warrant separate studies, I think Simone wished us to integrate these into a single program that we call the RED's III osmic Study. There are three aims, one of them is to look at a kind of concept of super donors with respect to are there polymorphisms in hemoglobin and iron regulation that might allow donors to both give more frequently without becoming iron depleted and potentially not fail as repeat blood donors and might those also be associated with predispositions to some of the consequences of iron depletion, such as Pike and RLS. The major thrust thought was to really drill into the genetic and metabolic differences that may

underlie storage related capacity.

So, in addition a major piece of this that's now being exploited through collaborations with various groups including speakers in this session, was to build a very large repository of samples derived from the donors of whose blood was collected and stored and is now being characterized genetically and metabolically. So the study involved enrollment of donors, the goal was 14 thousand donors and there were two phases -- a screening phase where we enrolled these 14 thousand donors and as you will see we subjected samples from these donors to storage induced hemolytic assay. The goal was to over enroll from minority population donors, so two thousand African-Americans, two thousand Asian, two thousand Hispanic, and also over enroll so called super donors. These were based on the criteria of greater than 10 donations in the prior two years without a hemoglobin deferral. So, all of these donors gave informed consent, including for extended genetic testing and long term storage

samples, and then, as you will see, samples were derived from these donors and a GWAS was executed on the samples characterized from these donors. There was also a recall phase where donors who demonstrated extremes of hemolysis and end of storage hemolysis on the screening phase were recalled, and the goal of that was to confirm that the findings from the screening phase were reproducible within donors over time, so after six months or so these donors were brought back and similar samples subjected to repeat storage hemolysis assays at end of storage, but in addition in that phase we required the whole unit which allowed for more extensive characterization.

So, this just shows that the testing was standardized between the blood center in Wisconsin and Tamir and Mark Gladman's group and the SRI which is the central lab or the program and there is a large central repository, and then materials being in process now for the genomics and the metabolomics is in progress now at Jim Zimring's program.

So, just in terms of logistics we actually looked at alternative and commercially bag systems, pediatric bags, for example, but we ended up having manufactured for the studies by Hermeneutics a bag that had identical plastic composition and volume to area ratios with respect to standard bags and then did validation studies that showed that when samples were transferred off of a leura reduced pack cell unit into these 12.5 ML transfer bags that the storage perimeters were identical or virtually identical in the parent unit and these specially produced transfer bags. So the release units were actually transfused into patients. We didn't consume these 14 thousand units in the screening phase, we couldn't afford to buy them, but in fact them being released to patients and prior and subsequent donations by these donors being transfused to patients allows us to now look at the clinical outcomes in our hub hospitals after the receipt of these units and correlate that with the in vitro and genetic and metabolomics data.

In addition, we acquired the leuk reduction filters and those were used to recover white cells for DNA analysis and there is an ample number of frozen white cells and DNA for future research. In terms of the testing we've completed a large (inaudible), I'll describe that, and the hemolysis assays were performed at end of storage on these transfer bag samples. I'll describe those in a little bit of detail and then again, the extreme hemolysis data from the different parameters that we measured were used to select a group of donors who were recalled for additional unit collection and that additional unit was stored and samples serially through the course of storage. We did also prepare transfer bag from this unit so we could validate the reliability of the transfer bag findings relative to the parent unit and findings. We had these four storage perimeters mechanical fragility ended up correlating strongly with, I forget whether it was oxidative osmotic and was less reproducible in this assay, so we actually restricted mechanical

fragility to the recall samples.

So, these are the assay sample storage hemolysis spontaneous breakdown of red cells and hemoglobin levels and supernatant. The other parameters were based on washed red cells and we performed both osmotic and oxidative hemolysis, again with a window of between 39 and 42-days post collection.

The study ended up accruing 13,770 donors who enrolled, some of them went through some enrollment, they consented but ended up not completing a donation and we got pretty close to our goals with respect to the minority population. We actually exceeded our goal with respect to high intensity donors. For some of the analyses I'll show now we excluded the high intensity because as I'll show you there is a significant confounding effect that donors who give more frequently have perturbed storage effects, so looking at some of the effects, such as donor age and gender, we restricted the analysis to a smaller group of about 10,500 donors excluding the high intensity

donors. So this just shows data that correlates sex and age with the storage hemolysis and this is similar to the data that Jason and Tamir published previously, so actually an interesting kind of hump in middle age males of an increased rate of storage hemolysis but significant, highly significant, differences between men and women. Here we are looking at osmotic hemolysis, again, significant differences between men and women, with again a sort of a middle aged increase in men, and then oxidative hemolysis again higher rates in men and women that disappear with the older age group donors.

This is looking at the racial ethnic categories, so if we just, for example, look as osmotic hemolysis you can see a significant shift in the overall histogram of correlations between levels of osmotic hemolysis and racial ethnic groups and in particular, African-American donors here, so a very significant shift towards lower osmotic hemolysis, more a capacity of red cells stored for 42-days from African- Americans donors

to tolerate osmotic stress.

In the summary table down here, just as looking at whether there is significantly increased or decreased hemolysis relative to Caucasian donors for these other donor categories, so you can see that African-Americans have significant increased storage hemolysis, decreased osmotic and increased oxidative. These findings were consistent with the hypothesis that minority populations, including both African- American and Asians, would be selected for polymorphisms that might influence storage properties.

A somewhat surprising finding was the observation that high intensity donors had significantly increased storage and reduced oxidative hemolysis. We'll talk about that a little bit more. In fact, here it is. This is just now including the high intensity donors, so we have fairly large numbers of donors that crossed this frequency of donation, so ranging from first time donors never phlebotomized up to donors who have given ten or more donations in the

prior two years and we see within both genders a highly significant reduction in oxygenated hemolysis with frequency of donations. This was a surprise to us and we suspect and are doing analyses. We do have ferritin and extended CBC data on all of these donations, so we suspect that this is at least in part driven by the fact that repeat donation results in iron loss, so this is just looking at the same population with respect to ferritin levels in obviously non-stored fresh blood collection and you see what's well known, which is with frequent donations you drop your ferritin dramatically. Women start out with a lower and drop to quite low levels, so we're pursuing the hypothesis that this finding with respect to frequency of donation could be attributable to iron depletion and essentially a sort of an iron deficient erythropoiesis. In a sense this is a little bit similar to iron deficiency anemia where the significant increased oxidated damage, et cetera, can cause red cells to hemolyze and iron deficiency.

So, this is data from Steve Spitalnik, presented at last year's ABB that makes the point that frequent phlebotomy and iron depletion could affect not only the donor's health but could affect the efficacy of the transfusions, which is sort of a potential observation from out study and what Steve did was essentially phlebotomize the iron depleted mice and phlebotomized them and demonstrated that when you transfuse blood from mice that have been iron depleted you see significantly reduced recovery of those red cells.

So this leads to the hypothesis that frequent blood donation may actually not only adversely affect the donors but may also result in a red cell product with lower transfusion efficacy.

These three perimeters that we measured were not correlated with each other, so this is good in that they are independent. We hope correlates of possible genetic pathways and metabolic pathways that will be analyzed in subsequent studies.

Now, as I mentioned, we recalled the donors with the extremes of hemolysis and the goal here was to validate that the findings from the index were reproducible on downstream donations and also to obtain large quantities of cells and material from the storage units for further study, so we did show a significant correlation, particularly osmotic and oxidative hemolysis. It is interestingly storage hemolysis, the FDA sort of standard for QC, has a very low dynamic range and relatively poor reproducibility within donors over time. Then this just shows the change over time in the perimeters both the spontaneous and the oxidative osmotic and mechanical hemolysis parameters and these data show again a classic progression in these parameters with the reproducibility within donors, the associations with racial ethnic categories, so we do believe that all these findings support the premise that there is a genetic component to the variability in donor propensity to store, and this has now been analyzed with the GWAS and metabolic component is

in progress.

Just a brief comment on the GWAS we started with a lot of research in terms of what was available, the UK biobank, and the interval study was using this UK biobank array, so we learned a lot from them, but we decided to go further and we developed what we're calling the Transfusion Medicine array. So we formed a series of expert groups and sought experienced collaborations with experts in blood grouping and sickle cell disease transplantation and iron metabolism, red cell metabolism, immunology coagulation, the interval study itself, and we created extended lists of all the known polymorphisms that are associated with all diseases and all pathways within these various contacts. So, we have a GWAS array that has been enriched and has a sort of major content of 350,000 snips that are classic full geno coverage array. We extended the coverage to improve representation of African-American and Asian populations as well as Brazilian because the same

array has been used on a large sickle cell cohort and the REDS-III program and them we added a large number of snips and copy number polymorphisms based on the expert panel input related to all of these areas that I alluded to. We also increased the representation across all of the genes that are known to be expressed and platelets and red cells, all of the genes associated with iron, all of the genes associated with sickle cell disease, and we ended up with a 100,000 snip and copy number variant array. This proved to be extremely accurate so it's been applied not only to the over 13,000 donations in the RCB all mix but about 3000 sickle cell samples from the RED's Brazil program, and just to point out here that we have 99.4 percent call rates across the gnome and lastly, looking at racial ethnic groups we have excellent ancestry calls with respect to the self-disclosed population racial ethnic groups and when we just looked at some known sort of associations for example, the calls with respect to the B or O or Duffy, ex cetera, classic blood group genes we

have minor (inaudible) frequencies that are very consistent with reported rates. So, we have a high confidence that this array is performing appropriately.

No data on the array yet in terms of the correlations with storage phenotypes or other red cell parameters but that is in progress at this point and should be available by early next year. Just to acknowledge this is obviously a huge team effort and working group, RTI support, lab team members that did all that testing and then the particularly NHOBI and the participating blood centers.

Thank you. (Applause)

DR. RAIFE: We were just discussing -- I think we'll just discuss this until a little after 1:00 o'clock, it will eat into our lunch just a little bit, but that way we'll stay on schedule. So, now this session is open for discussion.

DR. DOCTOR: Well, thank you, those talks were brilliant and really interesting. I have two questions. I'm sorry, Alan Doctor, Wash

U.

First, for the metabolomics pair, this end, I'm interested in your thoughts about the importance of examining the dynamic range in the pathways that you're studying. For example, versus just studying them at rest, and for example, imposing oxidative stress and examining the ability to respond to that in terms of defense and looking at the ability to accelerate glycolysis or reducing equivalent recycling and whether that's necessary to unveil weaknesses that may not be apparent when studying at rest?

DR. D'ALESSANDRO: Thanks for the question. If I got the question right you are asking whether we have any idea of the dynamic range of (inaudible) responses that are donor dependent and stress dependent. We've done several studies in red cell self-storage but also in responses of red blood cells to acclimatization to the altitude of that box here, or responses to external stressors or stimuli there is a chance, for example, severe hemorrhagic shock. It would

be great to perform (inaudible) on sort of rebel cells challenge with (inaudible) for example. We've done some studies on rebel cells that are aggravated with anti-oxygen like (inaudible) and vitamin C for example, that to some extent can also become pro-oxidants. I think that it will be key as Professor Palsson was pointing out before that at first for sole numbers we need to analyze many more samples from many more different donors than what he had done so far.

The second thing is we have to do that in reproducible manner meaning, for example, (inaudible) and the third thing, we need to make these available for the public audience through generation of data bases that are freely assessable and amenable to elaboration through system biology approaches.

DR. PALSSON: So many I can say a couple of things. I think you were interested in the dynamic ranges of the fluxes of these pathways that are operating in the red cell under cold storage. So, what I did not have the time to go through or describe is that these network level flux models that we have estimate, based on all the data that you have, what the most likely flux is to a particular pathway. Based on that we can -- and it's described in the publication -- we can look at what the state of these pathways are in the three phases of metabolic decay.

As an example, the non-oxidated part of the pentose pathways is quite interesting. So, in phase 1, the pentose pathway goes through out of glycolysis and back into glycolysis. In phase 2, the salvage pathway is very active and is recycling the pentose to bind it to rebuild the nucleotides. In phase 3 it reverses again into the same state that this was before. So, if you are thinking about dynamic ranges that pathway, the non-oxidated branch of those pathways flips

directions twice, or I should say, flips direction at each of the (inaudible) in the curve. So there is clearly a lot of dynamic range, at least in that pathway. I believe one of the things that you are quite interested in is also to see how much of the oxidative stressors restrict the total flux through the pentose pathway and it is interesting that when we started tinkering with the glutathione precursors some of those constraints maybe affected by the availability of glutathione. It is being turned over, clearly, during storage and one of these 8 biomarkers is also oxoproline, which is actually a derivative product from glutathione. So maybe we can also have some dynamic range in what flux is through that pathway under storage conditions. So, glutathione degradation. So, I guess what I'm trying to say is that some of the pathways that we know now to be operating the red cell there is quite a bit of dynamic range in them. I think others, you know, like the glycolytic flux just seems to decay continuously over time and maybe

the hydrogenates oxidation is one process that continually degrades glycolytic rate at the oxidative part of the pentose pathways also seems to also be going down over time. But some of the other pathways seem to have a little bit more of a dynamic range to them.

DR. D'ALESSANDRO: If I may add something, these models are also relevant in that of course taking into account key variables such as PH and the other thing is that we can use these approaches to model the way the red blood cell response, not just in the normal range, but also in some pathologic ranges. One of the questions that has been identified to be interesting for the community is, for example, whether glucose-6 is (inaudible) donors may be good donors and these models can predict at least from a metabolic standpoint whether you would have a given metabolic outcome rather than other, which we don't know whether it's going to be (inaudible) in the clinics or the final outcome, but it's something that we can do now.

DR. PATEL: Can I just follow-up on that question regarding the way the metabolic metabolomics data you're getting is helping us on this and how red cells handle different types of oxidative stress. So, a couple of questions. When you have the shifts in these metabolites and hence the metabolism, do you see changes in the activity of the enzymes that use those metabolites to protect the red cells against whether it be hydrogen peroxide lipid peroxides, and so forth, that's my first question. The second question, which is a little bit off the wall, that comes from a few studies published from a group in England a couple of year ago that suggest that there are circadian rhythms in anti-oxidant enzymes in red cells. So, have you seen or has anybody else seen any differences any whatever endpoint related to red cell storage and time of collection or time of day of analysis or things of that nature?

DR. D'ALESSANDRO: If I may go ahead, so the sample preparations strategies and sample

handling strategies deeply influence the metabolic phenotypes and the first question, again, was about --

DR. PATEL: What is the function of the change in the metabolic flux? What is happening to the proteins that you use as metabolites?

DR. D'ALESSANDRO: Interestingly, there is two things that we have been observing at least in our studies which doesn't necessarily imply that it's correct for every single observation, but what we're observing is that some glycolytic enzyme, for example, change the rates of activity and some enzymes actually start performing some moonlighting function. I'm thinking of, for example, anti-oxygen and enzymes such as peroxide oxygen 2 that at some point during that self-storage becomes a phospholipase or another enzyme such as lactate hydrogenase that at some point go into the reducing environment that, you know, is (inaudible) by lowering the PH, starts playing other functions such as, for example, converting ketoglutarate to all oxoglutarate in

the reaction that generates (inaudible). So, yeah, that is an interesting point.

Dr. BUSCH: I'd like to comment on a point here which is you talked about an endometabolome and exometabolome and in a sense in a stored red cell you have the fluid in which the red cells are suspended through storage and then you have the red cells with the membrane and internal cytoplasm, if you will, and some of your studies I know have looked separately at both, for the big study that we've executed we actually have done a lot of control work for metabolomics, et cetera, which I'm presuming you are aware of, but what we're doing is we're freezing the entire sample, which is a combination of the endometabolome and the exometabolome and I'm just curious how important is it to separate the vast majority of the fluid, if you will, in a stored red cell is probably within the red cells, but is it important to separate those and is you insights to metabolism going to be influenced by the sample that you start with and the way it's processed?

DR. D'ALESSANDRO: I think it definitely is, the point is you are going to ask different questions. At the end if you assume that you have potential markers like xanthine and hypoxanthine as a precursor of H202 in that you're regenerating pathways. Those are the level of intracellular (inaudible) xanthine and extracellular xanthine intracellular and extracellular may be combined to have maybe a better predictor of -- maybe Professor Palsson knows this better.

DR. PALSSON: So maybe I can say a few things about that. Some of the metabolites, like the purines that you mentioned, measuring inside and outside is roughly the same measurement. In metabolic phase 3 the purine nucleotides have begun to fall apart and some xanthine and hypoxanthine builds up inside the cell and leaks across the membrane and builds up there. So they show a similar profile but if you look at other compounds like adrenal glucose it's in high

concentration outside and they're have been up taken but once they're inside the cell like the glucose (inaudible) operates on it you won't see a buildup of glucose inside the cell but it reaches kind of a quaisi study state and that's very important when you are calculating the flux math. So sometimes it's important to measure them separately, sometimes it's not, but on the previous I just want to point out that this is the only state that generation and systems biology analysis is a top-down kind of a process where you start with the overall features and you go into more detail and once you pass the statistical analysis of how variables are related you can get into a pathway level of analysis to look at the relative flux through the pathways like I talked about and then even it can go into further level of detail but you are quizzing individual biochemical events. Observing these kinks in the overall pattern raises questions about first how is the flux match shifted within the different metabolic states and we've been able to map that

out, then you ask the next question: why? What is the mechanism? So, I believe in our transfusion paper we point out that there are five individual and sematic steps of particular interest in how they are regulated and how they may produce this overall pattern that we are talking about.

I mentioned one of them, the mutase that makes 2,3, DPG from 1,3 DPG. We always assumed that 2,3, DPG degrades by dephrosphorylation and becomes the 3 PG and just goes down the glycolysis but when we balanced the whole map and when we looked at the proton balancing the PH it is much likelier that the it actually goes back to 1,3 DPG and then goes down glycolysis and makes 2 ATPs and that's when you have the built up of ATP during that period.

Another enzyme that shows up in that analysis is GAPD hydrogenase and we haven't looked at that but this you saw with Angelo, he's been looking at the stability of that enzyme and how that may affect the overall metabolism. So, we

have a half a dozen very well defined biochemical hypothesis on the function and the regulation of these enzymes that probably warrant some detailed chemical work.

DR. BUSCH: Is the point on the circadian rhythm because, I mean, reality is a bag of red cells or the red cells that are circulating in our body, vary in age from being produced today to having them produced three months ago, one possible explanation for why women, for example, have different properties is that during their menstruating and childbearing period is they are essentially losing red cells and they may have on average younger population of red cells. So in all of these metabolic like studies do you in any way think it's important to discriminate the actual age? We're looking at an average phenomenon of the mixture of red cells that have around for three months versus those that have been produced today.

DR. PALSSON: Well, how do we start to think about that? We would love to be able to age

fractionate the cells in the initial collections to see if it decays at different rates, that's for sure. I just don't know how to do that. Maybe you do. But that would be wonderful if you could do that. But it is interesting though that even if it is a population of red cells that comes out of the donor first of mixed physiological age they seem to go to these three phase shifts as a whole. So, in that sense maybe the physiological age creates some variation on hold individual red cells perform but we don't see any evidence of subpopulations in there. There is that subtle kink though that I mentioned that is on day 32 to 35 that maybe warrants a little more investigation, but the overall pattern seems to be the same for all the red cells of the same physiological age. It doesn't exclude the error parts there if you were actually able to fractionate and maybe the slightly different quantitative pattern but the qualitative pattern is the same.

DR. D'ALESSANDRO: We did also perform

some analogies of rebel cell population and through (inaudible) gradients. We didn't store them. Rapid cell population it is non- (inaudible) it's 60's, 70's that have different metabolic phenotypes. For example, it is known that glucose 6 phosphate dehydrogenase activity and (inaudible) decline with the age of the red blood cell population and it is also true that the red blood cells are younger blood cells population from my young donor is not a younger blood cell population from an old donor. We are doing some of the tying of the (inaudible). I think that the experiment of the sorting the population and then preserving them and performing metabolomics and proteomics analyses will give our data a lot.

DR. PATEL: Just one last comment on that last bit of discussion. When we've tried that, when you just take stored red cells and age amount 35 days and look at their age based on physiologic age they all behave like older red cells and they look like older red cells based on their ability to be separated, but what's

interesting is that if you've collected fresh you see these great populations and you lose that resolution after the storage time in the blood bank.

DR. D'ALESSANDRO: I remember a (inaudible) just came out like two years ago where actually they showed that the preferential population of these lost at the end of the search field which is no more likely to be normalize at the end of the search period is the one that was the oldest population at the beginning of the search. The one with the extreme and differential density but I haven't seen any further study on that.

DR. RAIFE: I'd like to being up one last point. There has been expressed here sort of a yearning and need for a way to share data. I think we all agree that we have large volumes of data accumulating and not a very good way of integrating data from one study and one laboratory to the next. What are your thoughts on that?

DR. PALSSON: Well, I think it is an

issue that needs to be addressed and I know REDS-III is building a big data base but would that only contain data from that study or could it be open tops so that Angelo could put his data in and we could put our data in and so could others. So, I think the need for that data base is strong. I can tell you that I work a little bit with other organizations, the e-coli and the e- coli community has built data bases where they harvest say expression "pull in data" from every single laboratory that's generating such information and what's interesting about big data analysis of some data like that is not so much that you learn scientific things, you learn other things. For instance, you learn that data from the different labs cluster differently. So even if the procedures they use ostensibly are the same, there are some subtle uncontrolled variables in the protocols that are different and they lead to different properties of the data. Big data sets also have revealed other issues with other experimental protocols, so some people, for

instance, measure growth rates in 96-well plates robotically and end up looking at the data you see a few of the wells are always out layers, so there is something wrong with the reader do detect things from that well, so what I am trying to point out by these two detailed examples they've been irrelevant to the field of study here but is is the fact that big data analysis like that helps the as a whole to recognize the data point of differences in protocols and a number of other things in addition to being scientifically valuable.

DR. BUSCH: Certainly the Simone's step-down REDS RBC omics data set will one become a public use data set at some point in the future but while we're working on it we're inviting collaboration so Angelo is now involved and we've done the collaboration with you, Tom, to validate your ATP measurements and both on the analysis side, the sample sharing side, and the data sharing side, during the process will become an open collaborative enterprise and then in the long

run will of necessity be established as a public use data set.

DR. PALSSON: Yes, on that I think it's very important to have a data base like that that is open that there's discipline in depositing the data as it's generated because once you have the data in Excel spreadsheet that's three months old it most likely never will go into a database. Like I said, it becomes bricked, it becomes separate from the rest, it loses a lot of utility if it isn't flowing into the database basically real time or more or less real time.

DR. DOCTOR: Sure, this GWAS design question about the REDS-III omics project, I'm wondering if this consideration for epigenetic regulation of the gene array or the gene sets that you're exploring and whether there is enough known about that to consider the importance that might otherwise be missed and the way you're collecting the samples will there be an opportunity to evaluate that?

DR. BUSCH: Yes, so by epigenetic you

mean things like methylation of DNA or RNA transcripts, et cetera. Yeah, it's a good question, certainly the GWAS itself is a DNA based analysis we are doing the metabolomic component of both the serial storage units and we're talking with Angelo about doing metabolomics end of storage on the entire 13,500 samples so there are cells frozen but these are PMCs form these donors so you could do RNA expression but how relevant would that be to what's inside the red cells and influencing red cell storage?

I mean, most of the focus of the metabolomics is on the proteome or the metabalome but we now understand that there is probably 50 fold more genes in our genome that are being expressed to RNAs that are functional that are not even translated into proteins. Now, would those have any relevance to red cell storage phenotype? I don't think so. I mean these are relevant to the expression of the DNA and control of transcription, et cetera, and they may be genetically causing these epigenetic phenomenon

may be driving what we're seeing in different racial ethnic groups and different individuals but I don't think they're still operational within a red cell unit in a blood bank. What do you think?

DR. PALSSON: Epigenetic parameters? I don't know where to start on that one. But I will say that we will, I think, have different requirements for genetic data that comes with the particular cells that you're working with in an experiment. I don't know if there are ethical issues, you know, and all of that but it would be nice to have the genetic data. Of course, if you did deprotonic coverage of the data sets you will see the immunized sequel is different right there in the red cell proteome might be enough.

DR. RAIFE: I think we should break for lunch. Thank you. And reconvene at 2:00 p.m.

(WHEREUPON, at 1:12 p.m. a luncheon recess was taken.)

AFTERNOON SESSION (2:04 p.m)

DR. KLEIN: Well, if we can take our seats, we will start the afternoon session and the

first session one on animal models and our first speaker will be Dr. Paul Buehler. Dr. Buehler is a pharmacologist and a laboratory of biochemistry and vascular biology at CBER and he's going to be speaking about the potential biomarkers of red cell function in animal studies - Paul.

DR. BUEHLER: Thanks, Dr. Klein. So, I'm basically a toxicologist. I look at things in terms of dose and exposure. What I'll do is I'll go over some animal models, define biomarkers, give some examples of biomarkers as they might relate to red blood cell transfusion and then provide an example animal model that we use for transfusion and some characteristics of that model and how to apply biomarkers to that.

So this is the way I characterize animal models. I characterize them into efficacy models, which are basically proof of concept models. They evaluate animals with disease which is either spontaneous or existing, they typically have endpoints of diseases attenuation or reduced mortality.

Secondly, toxicology models. These are the types of models we deal with at FDA. These are preclinical safety assessment models. They usually follow GOP, they're designed to include rodents and non-rodent species and the importance in terms of difference between the models here, is that these are animals with a healthy background. They're all healthy models, no disease state, and they're designed to understand essentially the dose dependence of intervention and how that does effects clinical chemistry, hematology and organ function.

Combined models are actually quite useful. There's an example of those types of models in this meeting and these models basically have a disease state which essentially evaluate a potential additive effect of an intervention. They use mortality as an endpoint, typically, and they can be quite useful for assessing a mechanism of action and what toxicological agent was actually responsible for a type of event.

So, the way I see potential advantages

in terms of red blood cell evaluation for animal models is that they may predict safety concerns and toxicological response, they can help elucidate mechanisms of toxicity not easily studies in humans and they allow for comparative safety and efficacy between preparations. For example, what I show here -- a biomarker is essentially very strictly defined and it's defined by this group -- it's term best, not to type the best year associated with or known about, this is a biomarker's endpoints and other tools working group that FDA and NIH has developed and they have published their working group findings online, and these are available, so they define a biomarker as a characteristic that is measured as an indicator of normal biologic process, pathologic response or response to an exposure intervention, and this actually can include a therapeutic intervention, so you can have molecular, histological, radiographic, or physiologic types of biomarkers.

The examples that I thought might be useful for in- vivo evaluation of blood quality

are safety, or response biomarkers, which is a category of biomarkers, and then safety, which is basically toxicology and pharmacodynamics, which is basically efficacy.

So, examples of pharmacodynamics markers could be blood flow, tissue oxygenation such as arterial venous blood gases, HIF-1 accumulation, pimonidazole adduct formation, safety, if you use an example such as the kidney, neutrophil gelatinase associated lipocalin or NGAL, which is used both non-clinically and clinically. In animals you can look at the gene response, the protein response, and then potentially renal tubular necrosis.

So, we have a model that we use that is a Guinea pig base model. This model we essentially started using because it has similar antioxidant properties essentially to humans. Guinea pigs lack the final enzyme in the production of ascorbic acid. They have similar SOD-isoforms, they have similar catalyst, similar activities, RBC disc diameter in the Guinea pigs

is very similar to humans and duration of RBC circulation is very similar to humans. We can also use these models or these species as models of systemic blood flow and tissue oxygenation.

So, the way our model is basically set up is we collect blood, leuko reduce, separate, store, re-suspend, transfuse at 10, 30, and 90 percent which we equate to 1, 3 and 9 unit, then we analyze tissue for acute responses at 8 and 24 hours and here we look at biomarker determination.

So if we look at the characteristics of AS-3 stored Guinea pig blood it's pretty much at 1 day and then 14 days, it's pretty similar to what Bennett and Guerrero published in 2007 in PNAS for 42-day old blood, and in our model here you can see what's in the bag at one day, what's in the bag at 7 days, you can see the drastically changed morphological shape of the red blood cell echinocytes and the start of the formation of syrotocynic echinocytes, a decrease in deformability. If you take samples from animals that are transfused at eight hours, essentially

see disc shapes at one day and then at 14 days you some echinocytic forms and then in the spleen non-transfused you can see the changes in accumulation in RBCs.

So the example I gave of pharmacodynamics response, what I'm talking about here is potential to use something like blood flow in an animal, and this is a very simple approach, all we're doing here is looking at a laser Doppler flow probe around an artery like the aorta, so this is systemic blood flow, it's large vessel, and what we do is we increase the transfusion going in from 10 to 90 and with new blood or fresh blood here we can see the effect, with stored blood you see the effect here, so there's at 90 percent transfusion you see a decrease in about 50 percent with the stored blood.

The issue of tissue oxygenation, there is two potential biomarkers, HIF-1 alpha and pimonidazole, which one is endogenous and one is exogenous. HIF-1 alpha is typically the degraded under physioxic conditions, it's ubiguitinated,

it's degraded by the proteasome. Under inadequate oxygen supply the echinocytes and the cytosols then is translocated to the nucleus where it induces hypoxia inducible genes and then this can be measured by several different measurements.

With pimonidazole you inject this at a time point, usually an hour before you want to take tissue, when tissue PO2 drops below 10 millimeters of mercury the imidazole ring is activated this reacts with protein thiols and then you have adducts formed which you can either probe with Western blot or you can probe with immunohistochemistry, and we particularly look at the kidney because in the kidney there happens to be a nice gradient of oxygen difference and seen in the cortex typically the tissue PO2 is about 50 millimeters of mercury I the medulla it's much lower, 10 to 20 millimeters of mercury, so this provides a nice area for us to determine any differences.

So here are PO2, we can see with fresh RBCs no change after 8 hours, with stored RBCs

there is a decrease, same with venous, and here we see the change in HIF-1 alpha, so at 14 days HIF-1 alpha increases significantly, with stored blood at 1-day old blood, essentially, a little bit of increase but not much. If you look at pimonidazole similarly we can see these adducts after immuno blotting in the same area at 14 days of storage, and this is significantly increased from fresh blood, and then if we stain the tissue you can see an increase in the areas in the medulla that stain positive for immunoactivity with the antibody against pimonidazole adducts.

So the safety biomarkers that I suggested before was NGAL, which has use in an animal model as well as clinical use, so we know that free hemoglobin and iron are potential problems, so if we dose animals with, as I said, 1, 3, or 9 units, which is similar to 10, 30, or 90 percent transfusion we see an increase in hemoglobin which increases about 1.5 milligram per mil and when we use the hemoglobin binding protein haptoglobin we can bring in the free hemoglobin

down to baseline, and this is seen in the chromatography here with free hemoglobin and then with haptaglobin it's bound in a complex with non-transferring bound iron with increasing units of red blood cells we see an increase in NTBI and when we administer the iron binding transferrin and signaling protein apha-transferrin we can essentially see a decrease to basal levels of non-transferrin bind iron and this can be seen in the AUC calculation.

If we look at the dose dependence in 14-day old stored blood, so 1, 3, 9 units, here we start to see some tubular necrosis in the cortex. This is also associated with glomerular microvascular system micro thrombi, and if we look at the biomarker NGAL we can see a dose dependent increase in NGAL MRNA and then a dose dependent increase in the distal tubules which is where NGAL is produced when injury is created. And in both these cases when we administer haptaglobin at 300 milligrams per kilogram for transferrin we see a decrease in these effects and that just suggests

that iron and hemoglobin are playing partly a role.

The other issues we can ferret out here are from the other proteins that we see in the kidney; we can characterize these into categories. Here we characterize them into HB catabolism or oxidative stress response. With stored blood you see that these proteins increase quite significantly. Typically, renal tubular reabsorbs plasma proteins and these accumulate in the kidney after there is an injury and then we see an increase in these proteins on a proteomic analysis.

One other thing I want to show you is what we see in the vasculature. These are typically the two tissues where we see does dependent affects. We see increase with dose in nitric oxide consumption in the vasculature; we see an increase in plasma lipid peroxidation potential and then we start to see a dose dependent pathological increase at certain areas in the aorta, not the entire aorta, but we can

find areas in the aorta where we see a dose dependent increase in a particular necrosis which is called coagulative necrosis and then we see associated with that alpha smooth muscle actin decrease in these areas.

Again, this can be blocked with haptaglobin and apha transferrin and interestingly these areas where there is damage we see a larger decrease of iron deposition.

So, I will say that since starting to look at RBCs in terms of a toxicological affect, this is a considerably different challenge than small molecules or even protein based molecules based on the fact that you are essentially using surrogate cells from the animal itself and that's somewhat tricky because you're trying to correlate this to a human response. However, I think animal studies could be quite useful in understanding the quality of RBC preparations when we're doing comparative analysis between things like pathogen reduced stem cell derived or stored RBCs. We can identify biomarkers even in addition to what we

have, and these are just examples, that would apply to RBC pharmacodynamics responses as well as safety and these, in my mind, are both related to quality and in my mind it's feasible that these biomarkers for nonclinical evaluation could be determined which best translates to humans and then identified and validated and potentially animal models could be of use in assessing overall red blood cell quality.

I just want to thank the people in my lab, particularly Jin Beck and Ila, who have been extremely helpful and my lab chief, Abdu Alayash and my management structure at the FDA and my collaborators in Zurich who do a lot of the proteomic work in genomic work we do. Thank you.

(Applause)

DR. KLEIN: Thank you very much. Our next speaker is going to be Dr. Tim McMahon. He is the Associate Professor of Medicine at Duke and he's going to talk to us about correction of anemia, humanized and other mouse models.

DR. McMAHON: Thank you, Dr. Klein and

I'd like to thank the organizers for inviting me, it's been a great meeting so far and the stage has been well set by Paul and others as I'll talk about mouse models of transfusion and in particular a humanized mouse model of transfusion with its pluses and minuses.

So, we've talked quite a bit today already about the benefit and harm with red cell transfusion. One can think of their animal model as looking at one of these or the other, but preferably really the balance between the two with ultimately a kind of hard outcome like mortality in mice.

I think it's also important also to understand that the context for which the transfusion for anemia takes place, there is always a reason for anemia and it varies and therefore the interaction with the transfuse of red cells will also vary. Paul gave a nice example of some organ specific readouts, in his case the kidney, in our case the lung. I'll talk about modeling in both directions. Modeling in

the mouse in such a way that ultimately you are set to bring it back to the human. Again, also equate and impulse remarks.

One way I look at the red cell storage lesion, as I call it, is as being two baskets of problems. One, a loss of good things that the red cell normally needs to function, things like DPG for oxygen kinetics, ATP for a number of things nitrite oxide and its derivatives, like snow, and on the other hand the accumulation of bad things; Heme, hemoglobin, micro particles, lipid mediators in the supernatants, anti-leukocyte antibodies are an example.

When designing a mouse model of transfusion or using one it is important to keep in mind which sort of basket are we dealing with and also the interaction between the wo. For example, when you are interested in the role loss of ATP may play it may not be sufficient to transfuse 10 percent or 30 percent if the native red cells there are able to compensate you may have to move more toward an exchange transfusion.

So the context in which our transfusion model happens to be placed is in critical illness. This is an area where lots of transfusion still takes place in spite of the changes over the last few years, and importantly we know that in this population, like in others, anemia is in fact a positive risk factor for adverse outcomes including mortality independent of red cell transfusion. So, we know that even though liberal transfusion is no better than conservative transfusion, anemia is a problem here.

So, we looked at the literature with an eye toward what might be going on when patients fail to do better with more aggressive transfusion as a way to look toward how transfusion might be able to be improved. Looking at literature like the TRICC trial pulmonary sequelae (inaudible) you see this in a number of the clinical trials of liberal versus restrictive transfusion or storage for longer versus shorter periods. Things like pulmonary edema, ARDS, excessively present in the liberally transfused group. So we took that as a

starting point and some other considerations we've talked about are defining the condition where anemia and transfusion are present, it's important to have some sense that the biology in the mouse or in the mouse red cell mirrors that in the human, and I'll talk more about that, it's important to look for confounders or unanticipated consequences and it's important to look at relevant and accessible endpoints that you can then bring back to clinical practice or clinical question.

So, this is our model, maybe not the prettiest model ever to walk the runway -- with a face like that you might be surprised that this our model -- it's hair is not great, it's skins is not great, but we're using the nude mouse for transfusion of human cells in order to be able to study human red cells when transfused. The nude mouse has been used in a lot of applications. It lacks a thymus and lacks or has very few T lymphocytes and so you can transfuse foreign cells and not worry about rejection.

When using a humanized mouse model like this one important consideration is the difference in size of the red cells. The human red cell is a bit bigger than the mouse red cell shown to scale here and the difference in size between mouse and human can play out, particularly in the context in interaction with other changes, for example, the loss of red cell flexibility and shape change so a mouse capillary maybe about five microns actually similar to a human's, so both a mouse red cell and a human red cell will have to deform to get through capillaries in many cases but when those cells are older misshapen or less flexible now the size and shape together become something that leads to a phenotype.

So, we got interested in the use of the nude mouse model from our collaborator, Marilyn Telen, she and others have used the nude mouse for studies of sickle cell biology and they find that transfused sickle cells have a relatively short half-life in the nude mouse and shorter still once they're activated with epinephrine in this case,

whereas, AA normal human red cells survive, at least up to the 20 minutes transfused, and this data are similar to those that we know from human recovery studies. We haven't looked at longer time points and many of our studies take place in the first hour.

So, putting our mouse model together we are interested in the lung and lung function and blood oxygenation as an end point. There has been a lot of focus on blood oxygen delivery but less focus on blood oxygen uptake in the lung and that was of interest to us based on the clinical reports. In some of the experiments I'll show transfused what's equivalent to two units in a mouse and mice are normovolemic, they are anesthetized and breaking room air on a mechanical ventilator. We're looking typically at blood oxygenation and we are also tracking the fate of the red cells and we're interest in post transfusion adhesion of red cells. So illustrating that nude mice tolerate the transfusion of red cells shown here as a blood

oxygenation hemoglobin saturation tracing after transfusion of human red cells into a nude mouse, nice healthy saturation and it's relatively stable. In contrast when transfusing stored red cells, we see an early dip in oxygenation, not large, comes back to baseline and then this is kind of a typical thing that we see, it trails off a few percent lower over the next hour.

We're interested in the role of released ATP, red cells export ATP in response to a number of stimuli and this activity declines when red cells are stored, so we are interested in whether that might contribute to a storage lesion and in fact when you treat red cells with an ATP release inhibitor you see a similar drop in oxygenation and an early one followed by a later slow one.

Then we are interested in what happens to these red cells and what is going on in the lung, so we labeled red cells, transfused them, and then recovered mouse lungs after sacrificing the mice and find that again, with transfusion of normal fresh human red cells this six hours or

less after acquisition, stored and processed in a conventional manner, including liquid reduction, very little adhesion of the fresh human red cells, but after six weeks of storage these transfused red cells tend to adhere within the lung.

We've gone on to look at the mechanistic basis for this adhesion, both the storage induced adhesion and the adhesion apparently prevented by ARP release, shown here are experiments where we used an anti-body approach to try to identify adhesion receptors mediating the ATP sensitive adhesion of red cells and we find that most of this adhesion is attributable to ELW or ICAM-4 on the red cells, this is an antigen that is importantly common to mouse and human, so with the ELW anti-body we blocked that drop in oxygenation with transfusion and there was a trend toward a decrease in accumulation of red cells in the alveolar space. One of the phenotypes in this model.

It is important with key findings in humanized mouse model to validate using mouse red

cells. It won't always turn out to be validated, but we look to see whether mouse red cells treated with an ATP inhibitor were also adherence in the lung and also led to this impaired red blood oxygen uptake and extravasation into the airspace and we found that that's the case with mouse transfusion as well, so a drop in oxygenation with glibenclamide treated mouse red cells transfused into a mouse this is the ATP release inhibitor and extravasation of the red cells into the alveolar air spaces.

So, making the point that it's important to confirm key findings, at least, with a mouse-mouse transfusion model. We're interested also in the peripheral circulation and the ability to track some of these changes in red cell adhesion, in particular in real time, using intravital microscopy and in collaboration with Mark Dewhurst we have been using a window chamber model. I think we'll hear more about this model from Mark. We've learned many good lessons from him and his work, but essentially we are using

this chamber so this is a chamber implanted surgically on day zero and then the mouse recovers, the wound heals, and the mouse is healthy again three days later when we study it.

We're interested in blood oxygenation, again, as I mentioned, and oxygen delivery and distribution in the tissues and shown here are through the window chamber with intravital microscopy blood hemoglobin oxygenation maps, so we're seeing the microvasculature; we're seeing venules and arterials; we're seeing capillaries; and the color coding is hemoglobin oxygenation where the closer top red, the higher the saturation, and the closer too blue, the closer to zero saturation. So, you have a nice arteriovenous difference in oxygenation under normoxic conditions in these mice. They're anesthetized with a little bit of hypoxemia, then when we have them inhale hypoxic gas mixture 10 percent oxygen everything goes nearer too blue. So, establishing the ability to measure peripheral blood oxygenation and map it, we can get a little

fancier with these by also tracking red cells to indicate blood velocity within the arterial and venules. We can look at the direction of red cell flow at each pixel, and to the point of measures of tissue oxygenation that have been brought up earlier today, and Paul illustrated a nice couple of good techniques. Another one here that we can apply within the window chamber is a boron nanoparticle that has florescence quenched in the presence of oxygen, so you get a map of where PO2 is greatest within that window chamber. In this experiment we mapped hemoglobin oxygen saturation, PO2, and then looked at their correlation and saw them correlating nicely in this area that was well vascularized.

A typical experimental scheme using this model might have the mouse being pre-exposed to something to mimic a first hit in the two hit model of transfusion. Harm, endotoxin, for example, or LPS being instrumented; being human diluted to produce anemia -- perhaps critical anemia -- oxygen supply dependent anemia, rescuing

with a red cell transfusion and then while monitoring the mouse in terms of blood and tissue oxygen content, functional capillary density, red cell velocity, and then harvesting both the red cells and the tissues and organs of interest.

The human dilution model is titratable and it can give a mortality end point. Shown here is a video also examining the microcirculation, in this case of a human, during transfusion of 42-day old red cells done in collaboration with Elliott Bennett-Guerrero and this is using OPS imaging, you may also know it as Cytoscan technique, this is Orthogonal Polarization Spectroscopy. This is a measurement in the sublingual circulation with fairly normal flow that was in difference when a group of patients infused with 40 to 42-day red cells were compared to those getting 7- day old red cells, really there are great differences.

In contrast in the mouse, we have the nice advantage that we can label the red cells and see them much better. As shown here, again, an image from such an experiment. Nice, brisk flow,

arterial and venule seen together, capillaries.

Here is a video from a mouse getting human red cells that have been stored 35-days and you do see here is where the red cells are adherence, here, here.

Going further with this we are interested in non- invasive imaging in mice and humans at a little bit greater depth and in a way that will allow us to also read out blood oxygenation and potentially tissue oxygenation. Shown here is a video from a mouse using OCT, Optical Coherence Tomography. Can someone name the organ? The mouse is known for it. It's the mouse ear. So, the color coding here is depth and the more proximal structures are bright green, the more distal structures are orange and red. We're imaging at about a millimeter of depth with a width of imaging of a few hundred microns, and so getting a little deeper past the superficial vessels we think brings us closer to true biology, but again, the question of whether function capillary density, for example, or red cell flux

in the mouse ear is relevant is a fair question.

The same technique, OCT imaging that has the advantage of a less noise and greater depth of imaging, in this case the human hand.

So, in conclusion, the nude mouse model is useful to study human red cells and transfusion consequences in vivo, but that are certainly a number or caveats and I think ultimately key findings will need to be confirmed using mouse-mouse transfusion to rule out species differences, or red cell size differences as the basis, and also validated in larger animal models going forward, but it's very good for mechanism. The real time monitoring of oxygen delivery red cell flux and adhesion can be useful and relevant to transfusion outcomes and we thing that validating a non-invasive microcirculation imager that can be used in patients too makes sense.

Thank you. (Applause)

DR. KLEIN: Thank you very much. We'll now move from the mouse blood bank back to a hamster blood bank and we're fortunate to have Dr.

Marcos Intaglietta with us today, he's Professor of Bio Engineering at the Institute of Engineer and Medicine at U.C. San Diego and he's going to talk to us about the cardiovascular effect of a quart or a half-quart and two units of blood transfusion.

DR. INTAGLIETTA: Good afternoon. I was originally assigned to talk about the hamster microcirculation but thanks to Tim's wonderful lecture on the microcirculation of the mouse I am spared from that and, in fact, I would just repeat that and probably not so elegant as Tim did about the reasons for studying the in vivo microcirculation in this small animal.

Sometime ago, about three or four years ago, we became interested in applying the ideas of transfusion and particularly those related to the development of substitutes to treating anemia. For us, it is really an engineering goal. Engineers, one of the ways that they operate, is they make a pencil and paper model of something and they calculate and that way is a little bit

cheaper for instance to develop an airplane rather than build one and see if it flies.

So, we began by doing that to analyze what does a blood transfusion do. It was a mere mathematical model, a simple one, but we have done models of any levels of complexity. This model in particular is an elastic arterial system changes of volume, for instance, because of transfusion is accommodated in the venous circulation. If one increases hematocrit by a transfusion the reason increasing viscosity and furthermore is to change viscosity is the use of an elastic arterial circulation you will change blood flow and if you change blood flow you change the dynamics of the diffusion of oxygen out of blood because if oxygen moves slowly there is more time for oxygen to diffuse out according to the partial pressure of oxygen (inaudible). It is not a major effect but it is there, so it has to be accounted for because of the

(inaudible) 10 percent effect. So, the first point of doing a model

like this is to see what do we know about the viscosity of blood in anemia. In this particular case, in man, and as you see a lot is known about the situation of high blood viscosity with high hematocrits and very little is known about the situation of blood viscosity anemia, and in fact, many of this data is from the sixties - seventies era in which viscometers were not really all that good to measure this kind of fluids. So this is a sort of under populated and dated information.

However, the main message here is that the viscosity increases very rapidly as the hematocrit changes. It is a very non-linear clue for hematic curve. If we apply this data and calculate for man what the systemic oxygen delivery should be, and figure all the effects of viscosity on flow, we see that as for very small changes of hematocrit there are significant changes of blood flow and when these are translated into oxygen deliver at the rate of which oxygenated red blood cells come into the

microcirculation, if you are in this completely pathological area of hematocrit there is an effect. Blood transfusions to treat anemia occur in this range of deficits, hemoglobin and red blood cells, and therefore the gain that you can get is very little, and actually if you go beyond about a 50 percent deficit of red blood cells it is negative.

Well, when you applied this and look specifically at what blood transfusion does you've end up with this result here. If you have no deficit of red blood cells and if you had half a unit all the way to three units you decrease oxygen deliver and you only begin to be neutral if you have an oxygen deficit of 50 percent hematocrit, hemoglobin is half of normal and finally you have some gain but it's not very much. It is

percent over the anemic condition if the deficit is 60 percent, which is a hemoglobin of about 6 or thereabouts.

Now, we're engineers so we do models a

lot of times and to be honest, most of the time we fail. There are more failures that gains. But, the failure of a model, particularly if it is a big failure, is very interesting because it may mean that we've overlooked something. So, now, how are you going to find out? Well, you put the airplane in the wind tunnel and find out if it flies. And, so, we did that with the hamster. We made an anemic hamster and with 50 percent deficit and let it rest for a day and (inaudible) and we transfused blood -- hamster blood. And this is what happens. If you transfuse just plasma you get an increase of cardiac output of 40 percent, and of course, oxygen delivery goes down because you diluted the existing blood. If you transfuse a quarter unit of blood, you get just about, maybe a little bit more, oxygen delivery and of course you gained a little bit now because you actually added red blood cells and you keep going and finally you add two units of blood and now you have not really much of an increase in blood flow cardiac output but you have a fairly good increase

in oxygen delivery. If you use high viscosity plasma expanders, one unit to jack up the viscosity of plasma, well, you do fantastic. But this is a very old story for us, we have been acquainted with this for a very long time, the advantage of doing this is you're going to have to use red blood cells to really get the anemia a very strong change in oxygen delivery.

Okay, what is going on? The flow is supposed to be going down, and it goes up 40 percent regardless of how much blood you add. Well, it took a while for us to figure this out but we realized at some point that if you measure the endotoxins in blood in the normal animal in anemia this anemia formed by iso-hemodilution with human serum albumin, really not much happens. So, it is not that we have some contaminant in the laboratory, if we measure the classic markers for inflammation there is a significant increase. Now, I am told by experts in the field that nobody really gets extraordinarily excited by an increase of TNF alpha or IL-10 in this range here. But, it

is there. There is inflammation. The name of the game here is that we count this inflammatory process, the blood transfusion does appear not to have an effect. Now, Dr. Klein recently published a wonderful article saying correlation or not causation and this could be cause correlation. But we pursued this but still we haven't answered the question. But if it is inflammation you can give an anti-inflammatory and the effect should go away and we used dexamethasone, we administered the dexamethasone one hour before transfusion and then looked at what we usually look one or two hours, two hours is our favorite, and you see that the flow effect is half or less.

The summary of what is going on here. This is the theoretical curve for what should happen with treating anemia with blood transfusion. If you are here and you add red blood cells you should go up here. Now, that is the next paradox of transfusion. Even though theoretically you should be here, if you go and measure blood flow and oxygen and all the

parameters to characterize anemia, you're here. Now, this is experimental, so I'm going to add.5 or or a quarter,.5 one and two units of blood. In theory, because of the increase in blood viscosity I should go down this line here. In reality if I go on measuring there I go up this line here. There is a phenomenal effect due to, apparently, inflammation.

Now, this again, is what the viscose from an expanders' view might have added a very recent contribution, a very novel concept, that is still very novel in our hands so working through the mechanics of this, these are nano- particles that release nitric oxide and as of now one equivalent unit is also very difficult to establish what nitric oxide release compared to the release, for instance, from endogenous high viscosity due to sheer stress, how to compare the two. But we got here, so far.

This is my colleague and friend from Albert Einstein, Joel Friedman(phonetic), who is here in the audience and is the developer of this

very exciting transfer.

These are very preliminary conclusions, ideas, considerations, if you want to call them, or hypothesis. First, of all, if you're going to transfuse, you transfuse a quarter of a unit and half an until, one or two, while considering the very ability of the data to get the salient fact.

You all may know, I'm Italian, more or less. Now, if this is inflammation, there is probably a

limit of what inflammation can accomplish and if you are dealing with a sick, wounded patient that has lost blood he is going to be inflamed and therefore, the likelihood of getting a 40 percent increase just because you jacked up the inflammatory response is less. So there is going to be a limit to what you can accomplish with a blood transfusion which might account for why the transfusion sometimes is not effective.

And, then I'm sure that this question is in everybody's mind: but how about red blood

cells that are stored, that are older? Interestingly, if you look at all the data together it's identical to the fresh red blood cells. I have heard that comment several times here before today. There is one small change, the very ability is very high and from the transfused stored red blood cells, and in this case we used 14 days, which is the 42 days for man and hamsters, we got zero effect, no increase in blood flow and we got 100 percent increase. So, there is something going on there, at least in this model, and under these conditions that makes a difference for blood transfusion and for storage.

Thank you very much. (Applause)

DR. KLEIN: Thank you. It was very interesting and stimulating and I hope we'll have some questions about that. So, our final speaker for this session is Dr. Harold Swartz, who is Professor of Radiology at the Department of Radiology at the Geisel School of Medicine at Dartmouth, and he's going to tell us how to measure effective oxygenation of target tissues.

DR. SWARTZ: Thank you very much. And, I really appreciate the opportunity. This is a field that's not my field. My field is oxygenation, and I've learned a lot in, clearly, a challenging and important field, and, hopefully, my remarks will help a little bit.

So, I'm going to talk about, actually, how do we measure the effectiveness by -- and the hypothesis is that we want to measure the oxygen in the tissues, and, therefore, that's our target. So, I'm at the Geisel Medical School, which doesn't make much sense. Why would you have a Geisel Medical School? Until you realize this is our source of our funds, and, actually, there is something -- especially in modern medicine now -- to be at the Dr. Seuss Medical School. I think it tells us a lot about the state of things.

The disclosures -- we have a company that tries to make these instruments. It's not a very good company in which to invest, because any money that we make we put into the EPR Center, so I advise you not to invest in it. But, I have to

tell you that we're doing that. The other disclosure I have to tell you is that I'm really focused on oxygen. I see the world through an oxygen lens, and you should take my remarks with perhaps a grain of salt.

So, how do we measure? Our goal is to oxygenate tissues. That's why, I assume, we're transfusing, and, so, perhaps the way to do it is to look at what we're trying to oxygenate. I think it's important -- we learned this lesson in the hemoglobin derivatives -- is that you really need to make the measurements in the type of population for which this therapy is intended.

And, that probably means that different preparations will be useful for different circumstances, and especially between sick people and health volunteers. And, those are healthy people that other (Inaudible) have acute anemia from trauma. And, I think that's important to keep in mind.

You'd like to be able to make these measurements for the initial evaluation, and then

you'd like to be able to follow them in individuals. And, I think that's terribly important as we individualize medicine, as we recognize the need to individualize medicine, that you'd like to think about ways of making the measurement that you can do, actually, in the individual. Because, we're not smart enough to know what's good for everybody.

And, it's desirable to get this information dynamically and repeatedly, and, so that's what we're aiming for. And, if you look at what's available, if you listen to some of the discussions that we've had already, most of the techniques tend to fall short of these goals.

So, I have, as we often do, and one of the great things about computers now is that I rewrote my talk on the basis of some of the talks at the beginning. I had a little bit more clinical applications that I realized I had to modify to talk about, pre-clinical, which I did.

But, I think one of the themes that came out is the need for real data. This is such a

complex field. We can have lots of theories. We can have lots of principals. Given a circumstance, we can come up with an explanation for anything.

And, the thing that kind of destroys the conversations but does help is to have some data. And, I think the measurements of oxygen comes under that. It doesn't mean that the other measures, the functionality are -- and pathophysiology aren't important, but, and even the tissue oxygen isn't as important as the outcomes.

So, how do you actually measure oxygen in vivo? You need to think about the methods in terms of what they actually do. So, there's a few methods that actually measure oxygen. There are other methods that measure oxygen in the vascular system, which is quite different than the oxygen in the tissues. There's a lot of transport involved.

And, there are methods that measure parameters that reflect oxygen via plausible

measurement -- by plausible mechanisms, but they're not measuring oxygen. If you understand the mechanisms, if you understand the circumstances, these could be tremendously useful, but they're not measuring oxygen. And, I think you need to think about these things.

We're especially interested in converting these measurements into clinical practice, to change clinical practice, since you have to worry a lot about can they really be used. And, it would be nice if we had methods that we can use in animals and in humans and therefore translate the data from the animal models.

So, the methods that measure via plausible mechanisms are the hypoxic markers they talked about. They certainly don't measure oxygen. They tell you qualitatively whether or not there had been a time of hypoxia when these were delivered. There are some nice indicators of redox state.

You can get some indication by looking at metabolism using PET, BOLD MRI, will tell you

how much the oxyhemoglobin is around. And, that can give you some information, but you need a lot more to go from that to oxygen. Similarly, MRI has some powerful perfusion and diffusion measurements and increasingly MRI spectroscopy of hypoxia-related molecules.

These are all very useful techniques, but you have to understand what they're giving you. These are available clinically and pre-clinically, and we tend to use them, but the method -- we need to understand in what circumstances do they really tell us the oxygen itself.

So, there's some methods that measure oxygen in the vascular system, especially near infrared and blood gases. This is very useful information. They're telling you what's in the vasculature. You'd actually like to know more about the compartments of the vasculature. These are also available clinically and pre-clinically, but they don't necessarily relate to the oxygen.

So, what methods are there for measuring

oxygen in the tissues? And, the list is unfortunately not so great -- the oxygen electrode, the OxyLite, the fluorescence. I'm going to go through these one by one. There is a fluorine hydrocarbon NMR technique, and there is something that you've never heard of, but I make my living doing EPR oximetry, and so you'll hear a little bit about that.

So, the oxygen electrode is really considered the gold standard. Most of the data that we have in humans and much in the animals that is really measuring oxygen is done with this needle electrode that's passed through tissue, makes a pathway, and so it has some modest limitations, because you're injuring the tissue at the same time that you're making the measurement. You're sticking the needle through.

It has given very valuable data, but you can't use it over and over again, because you'd start chopping up the tissue, and that seems to be not a real good idea. And, it's no longer commercially available.

The OxyLite, the fluorescence quenching, oxygen quenching of the fluorescence emission is a very powerful technique. It can be used just like the oxygen electrode. It makes a pathway. It has the similar problems of (Inaudible -) local perturbations. It gives a very robust measurement with that limitation, but unfortunately it's not clinically available and it's not likely to be clinically available in the near future.

So, fluorine hydrocarbon NMR -- so, you inject this oxygen-sensitive material directly into the site. You can make repeated measurements over time, but somehow it's never had clinical translation, for a variety of reasons. Something to keep an eye out, -- they're not ready for prime time yet -- is there are some promising other NMR techniques that are sensitive to oxygen, and they may become available in the near future.

And, then finally, a little bit about EPR oximetry. It requires a one-time injection of oxygen-sensitive material. The thing that we use clinically right now is India Ink, the same thing

that your kids use in their skin. And, because of this, the FDA has granted a grandfathering of it so that we can use it directly and avoid the small perturbation of getting a drug through the few years and the few dollars that are involved in getting a new drug qualified. We haven't had to do that.

Once it's in there, the one-time measurement, you can then make measurements over time and you can make repeated measurements over time. It's been widely used in pre-clinical models successfully, and we're doing clinical measurements using both India Ink and PDMS, enclosed other materials so that, again, we can bypass the need for clearance of injecting material directly into tissues. And, this just gives you an idea of what this technique looks like, the -- oh, here's this.

So, basically, in the presence of oxygen -- in this case you see a squiggle. It doesn't matter. What it means -- you compress, it gets narrower. That means the oxygen is gone. That

is, you put a blood pressure cuff on the leg, you release it, it gets there, you can convert that directly into the oxygen.

In a robust way this is what it looks like physically. So, here is a foot with a bit of India Ink, or in the metatarsal head. Those of you who are involved with peripheral vascular disease and diabetes, this is where the pathophysiology of diabetes is, and wouldn't it be nice to be able to actually measure the pathophysiology directly. And, it takes about 5 minutes to do the measurement.

This is just to show you some data. There's some scatter here experimentally. These are years, so this is the injection in the same foot. So, once the material is in you can make the measurements for peripheral vascular disease, presumably indefinitely. But, we only started 10 years ago, so that's the most data I can give you.

So, this technique, EPR oximetry, has been used in virtually every tissue, from mice to pigs as well as in humans. We've now made

measurements in human subjects at several institutions in the United States -- Dartmouth, Emory, Yale, Rochester, Dana-Farber, and in Korea, Japan, and Belgium.

And, we've shown in humans now, and particularly with the help of NCI who's funded a program project, to make these measurements in cancer. And, we think one of the uses may be to tell you when more widely used things, such as NMR, will actually give you the parameter that you're interested in. So, I've taken you through very quickly, but I think I've actually stayed on time, which is not my usual case.

So, I just summarize, again, that I think the evaluation of the utility of the red-cell preparations should include actually measuring their effect on oxygen, and it should be including the subjects or the pathophysiology for which the therapy will be applied. This is a technical challenge to do with high competence, but I think there are techniques available and I'm highly prejudiced that I think EPR is a method of

choice that can be and should be used pre-clinically and clinically.

And, we see in humans and we see in the animals that everything isn't equal, and it's really useful to make the measurements in the individuals. And, so you'd like to have techniques in which you could use clinically to look at whether or not an individual is responding to the therapy and how much therapy you need, and then finally if, in fact, anybody is interested, we're really very glad to extend our collaborations. So, I think I should stop here and we'll go to the discussion. Thank you very much. (Applause)

DR. KLEIN: If I could ask the other speakers to please come up and have a seat at the table. And, others, please make your way to the microphones. And, while you're doing so, let me ask the first question. Have you done any studies in transfusion in animals at all?

DR. SWARTZ: It's an obvious question.

DR. KLEIN: It is an obvious one, not

only for standard transfusion but, again, for trying to get the issue of quality of the red cells and whether different tissues are oxygenated in different situations, different diseases. Questions from the audience.

DR. PATEL: I guess this is a question for Paul. In terms of the model you use, which obviously mimics the human situation because of the ascorbate issue, in comparing the data you've got compared to those from the literature or maybe things that you've done directly, using animal models that do synthesize their own ascorbate, is there a big difference in terms of stored red cell or transfusion toxicity, and if so, what --

DR. BUSCH: Actually, I don't know. You know, we started using that model because we were looking a lot at oxidative stress, and it seemed logical to use the model. But, we continued using it because there were some characteristics of it that seemed applicable to a transfusion. But, I mean, there are interesting characteristics that we can find that are different than a species like

a rat, which does produce ascorbate at large extents, and when it gets stressed it produces even more.

So, if you're looking at oxidative stress, I think, you know, small rodents are not ideal in terms of looking at mice or a rat. I mean, if you want a rodent and you're looking at those types of things, I think a guinea pig would be probably more ideal.

DR. KLEIN: Phil?

DR. SPINELLA: Phil Spinella, Wash U. Tim, with your new mouse model, how many different ways can you put it into shock, other than, I imagine, hemodilutional anemia? I realize with it being a SCID mouse it might be a sensitive animal, so you probably can't make it septic. Can you traumatize it and make it bleed to go into shock? I'm just wondering how many different ways can you put it into shock?

DR. MCMAHON: I think every way you can think of. I can't think of a model of shock that's been used in other animals that couldn't be

used in a mouse. It's not what we do, but, -- trauma, hemorrhage.

DR. SPINELLA: I just heard that (Inaudible -) have had a hard time keeping the mouse alive, period, if we made it septic, for example.

DR. MCMAHON: When septic?

DR. SPINELLA: Yes. I guess, have you seen this type of model used in other ways other than the way you're doing it now with dilutional anemia?

DR. MCMAHON: You're asking about other forms of anemia?

DR. SPINELLA: Are there any etiologies of shock in that model that you presented?

DR. MCMAHON: It's been used by others for hemorrhagic and septic shock.

DR. SPINELLA: Okay. Thank you.

DR. KLEIN: I have two questions for Dr. Buehler. First of all, the haptoglobin that you used in the hamster, was that human haptoglobin or was that hamster haptoglobin, and did you have any

issues with it?

DR. BUSCH: That's human. But, it's a single dose, so you're not going to see much in terms of immunogenicity. If we did repeated dosings, we could have a problem with that.

DR. KLEIN: And, the second question I had is that you saw renal toxicity in this model. We've been working with a dog model, as you know, and haven't seen any renal toxicity. Do you think that that's specific to the model, or are we doing something wrong?

DR. BUSCH: You're not doing anything wrong. Actually, we worked a lot with dogs, and we actually did a study where we transfused one gram per kg over 8 hours and saw absolutely no change on renal biopsy, no change on CT. I think it could go back to the issue of -- the dog has a very high level of haptoglobin, so that can also be an issue. And, they have, also, a very high level of ascorbic in the kidneys. Those could all be things that make them somewhat resistant to oxidative stress as it relates to hemoglobin as it

filters through the kidney.

DR. KLEIN: I have a question for Dr. Intaglietta. You moved from your model to your experimental design and then you said that if you used stored cells, 14-day stored cells, you just saw more variability but you didn't really see any differences in flow in oxygen delivery. So, do you think that this could be in any way used then to assess the quality of storage components, or is that simply not sensitive enough of a model to do that?

DR. INTAGLIETTA: I think under these conditions it is not sensitive enough, really.

DR. KLEIN: Is there a way to tweak it, do you think, so that you could use it specifically for that purpose? Because, it seems like it has a lot of promise in other ways.

DR. INTAGLIETTA: Yes. Mm-hmm.

DR. KLEIN: Larry.

DR. DUMONT: I'm Larry Dumont from BSRI-Denver, formerly of Geisel School of Medicine. So, actually, about 5 years ago we put

in an application to MBF for an animal infusion model. He was in the EPR system. He didn't gain any legs, unfortunately. But, Hal, maybe you could comment on work that you've done or your team's done in measuring oxygen in the tissue in the animal and repeated measurements in the systems, using indicators other than India Ink.

DR. SWARTZ: Yeah, so, we have some material that is, in fact, just a better sensor than India Ink. So, we can use India Ink readily in patients, and so that's the first that we've done, using something called lithium talasynin (Phonetic). We can measure differences of one tore (Phonetic) with pretty good accuracy, so it extends down.

We particularly looked at ischemia reperfusion injury in the brain. We've looked at ischemia reperfusion injury elsewhere. We've looked at liver. We've looked at kidney, following the medullary versus the cortex oxygen in the endotoxic shock. And, there's a number of

other -- ours is the only group that's doing humans (Inaudible) collaborators. But, there are a number of other really excellent laboratories, including one here at NIH that are doing EPR oximetry in pre-clinical models.

DR. MCMAHON: A question about the ink. Can you tell us what it is in the ink that binds or reacts with the oxygen?

DR. SWARTZ: Sure.

DR. MCMAHON: And, what I'm wondering is, are there other molecules that may light up the probe -- reactive oxygen species or nitrosative species?

DR. SWARTZ: Yeah. So, --

DR. MCMAHON: Are there any other caviats?

DR. SWARTZ: Sure. No, it's a good question. So, EPR just is looking at unpaired electrons. It's looking at stable free radicals. The reactive oxygen species are in an enough concentration so that they don't give us a problem. So, one of the strengths of the method

is that it's very specific.

One of the weaknesses of the method is it's very specific and it means you have to inject the material, which you'd rather not. You know, you'd rather use NMR which uses water, which is genius, isn't it -- 102 molar. What a nice concentration with which to work.

So, we have to inject -- the unpaired electrons in the India Ink just have to do with the carbon particles. They have some unpaired electrons that are sorting around. It's a magnetic resonance technique. The presence of oxygen -- oxygen has two unpaired electrons, because that's its ground state, and it's these -- that's also what makes it a good oxidizing agent, those unpaired electrons. And, it's just the physical interactions.

So, the oxygen is acting like a little magnet that is perturbing the magnetic field, and you end up getting a change in the line with -- that is proportionate to the concentration of oxygen. So, it's nice and robust, but you have to

put the material in.

DR. KLEIN: Before I call on Dr. Vostal, I want to follow up on that. So, it's a single injection and then you can make multiple measurements over time. Do I understand that correctly? Or, do you have to inject every time?

DR. SWARTZ: No. So, once you inject it's there forever, or generally forever, both the India Ink and the material that we put in, PDMS, which we're, in fact, going through an FDA IDE for approval and we've gotten our first six human subjects done. And, the idea is that it stays there. And, we have another technique for going deeper for an implantable resonator.

DR. KLEIN: Dr. Vostal.

DR. VOSTAL: Thank you. This will be a question for Tim. You mentioned that there's a size difference between human red cells and the mouse red cells. Have you noticed a difference in oxygen delivery in your model between the two cells?

DR. MCMAHON: Difference in oxygen

delivery? No. No, we haven't. We have seen some subtle differences in some of the phenotypes. For example, the post storage transfusion lesion phenotype between mouse and human that were otherwise unexplained and we thought might be kind of a combination of the biology we've described and also the size.

DR. VOSTAL: But, fresh versus fresh -- mouse, human? There doesn't seem to be a difference?

DR. MCMAHON: Oxygen delivery in the basal state?

DR. VOSTAL: Yes.

DR. MCMAHON: No, no difference.

DR. KLEIN: Dr. Doctor.

DR. DOCTOR: I just have a question for Dr. Intaglietta. Functional capillary density in your models. I'm curious. It's very interesting data in the window chamber, and I'm not familiar enough with it. How generalizable is that to vital organs, say brain, heart, kidney, liver in regulation of functional capillary density in

those organs? Have you been able to study that as well, and do the results in your window chamber, in the skin or subcutaneous muscle, you know, similarly reflect what's going on in the vital organs?

DR. INTAGLIETTA: Again, the question, please. I'm sorry. I hear so poorly that (Chuckles) --

DR. DOCTOR: The relationship between functional capillary density evaluation in vital organs and in the skin window chamber in your model. Have you been able to study functional capillary density in vital organs, also?

DR. INTAGLIETTA: Not really. The study in whole organs has to be done with microspheres and things like that. It is very, very difficult to correlate, because functional capillary density in the chamber is measured visually by a trained observer. So, it's a very accurate measurement. The microsphere technique is blind, so it's very, very difficult to make a correlation to it.

DR. KLEIN: If there are no other

questions -- Tim, you have another question? Go ahead.

DR. MCMAHON: Marcus, I'm really curious about the blood flow effect that you described with the high viscosity and also the red cell blood flow effect with the inflammatory cytokine profile. Have you done anything to try to pin down the basis for that -- things, for example, like residual leukocytes or free hemoglobin?

DR. INTAGLIETTA: At this point, what I have presented is what we have. We don't have a mechanism

(Inaudible) are produced. And, as I alluded to by mentioning Professor Klein's statement in his paper, we still have to treat it as an association, not causation.

DR. MCMAHON: Are the red cell units leukoreduced? Do you use a leuko filter for the red cells? Do you filter the red cell units to eliminate leukocytes -- use a filter? I can ask you later.

DR. KLEIN: We're at the hour, so I want to thank our speakers. Terrific session. And, if there are other questions, please ask them during the break. We're on break now and we'll be back at 4:00. Thank you. Thank you both. Thank you all. (Applause)

(Recess)

DR. SPINELLA: All right. If everybody can go ahead and get seated. We'll go ahead and get started with the second part of Session 4 on Animal Models. This Animal Model Session is gonna focus more on shock and trauma resuscitation. And it's a great pleasure for me to introduce a friend of mine for ten years, almost exactly, Mike reminded me today. Even though we worked in San Antonio for two years for the Army, I met him for the first time in Israel ten years ago. Mike is going to talk to us about swine models for shock trauma and resuscitation. And he is the Chief of the Damage Control and Resuscitation Program at the US Army Institute for Surgical Research.

DR. DUBICK: All right. Thanks. Good

afternoon. I'm glad to be here. So what I've been asked to talk about today is some of our swine models for hemorrhagic shock resuscitation. And this is our usual disclaimer that I'm not here as an official spokesperson for the DoD or the government. So, what are our goals for when we develop these animal models? We want to ensure that we have a relevant to hemorrhage and injury severity. The last several years we've also been interested in what's the coagulation status of the animals as we develop these models. We want the model to be able to help so that we can quantiate blood loss easily and we can also look at survival time. So we're looking at outcomes. We want to maximum it's reproducibility again related to survival. We would pretty much like the untreated animals to be a 100% lethal. And we want to want to minimize any artificial bias that may favor evaluation of a specific product. Cause we're a military lab, our focus is mainly on initial resuscitation and we want to make sure the model allows for inclusion of appropriate controls. So

the assumptions we make is that we the primary patient we're trying to treat is going to be a young, healthy, military casualty, who has a major life threatening active bleeding and at the time of injury they would have a normal coagulation system but this was going to change over time. Because the pig has a contractile spleen, we do a splenectomy on all our models and so we consider laparatomy we do for the splenectomy to be kind of an added stress as part of the model. And the resuscitation fluids that we evaluate are primarily those that are either recommended by the Committee on Tactical Combat Casualty Care, that are available and used by the medics, or other first responder in the field. And any adjuncts that we may add to the resuscitation fluids have been shown to be beneficial in rogue models. Or some other small animal model. So our common endpoints are survival, blood loss, fluid requirements to maintain blood pressure, coagulation variables, and we do thrombolastgraphy. Hemodynamics and metabolic

variables inflammatory models. And basically the model selection is dependant on the research question that we're trying to address. We typically have used immature female Yorkshire swine, or intact male Sinclair mini pigs in the 30-50 kg range. We do controlled and uncontrolled hemorrhage. Several of the studies have been hemorrhage alone, but to be more of a clinically relevant, military relevant model. We've added some poly-trauma including femur fracture. As I mentioned, we do splenectomy and depending on what the IOCOOP recommends, in our attending veterinarians. Animals, in they are anesthetized are ventilated with FI02 varying between 0.21 to 1. But we also have done conscious sedated models as well. And if they are anesthetized, they are typically heavily instrumented. The models have been used for comparison for resuscitation fluids previously colloids versus crystalloids but most recently blood products and related drugs including TXA. And benefits of small volume resuscitation was cydo- protective antioxidant

immune modulating therapies. And the military limited availability of fluid far forward, we focused on hypotensive resuscitation. So we've selected the swine cause there's been good evidence that swine behave similar to humans in response to blood loss and what I show on the slide on the right, on graphs on the right, is these are three separate hemorrhage models and basically around 50% blood loss, the animals will die. And it's similar to people to losing half of their blood volume, it's hard to keep them alive. And so, the animal seems to be a relevant model, similar to a human response to hemorrhage. The other nice thing about the swine models is that they can be part of multicenter trials. And this was a model that we used that was performed by three different centers. That mimic the treatment in humans, where they have a baseline, we do a femur fracture, that's the injury phase, then did a controlled hemorrhage and hemodiluation to get the animals to get coagulathapethic. And then they underwent a grade five liver injury, following by

treatment. And just showing some of the results. This case we were looking at fresh frozen plasma versus a lyophilized plasma, a dried plasma product is high relevance for the military, and so this multicenter trial allowed us to get results a lot faster. And basically, showing that this particular model worked in the sense that if you look at the post resuscitation blood loss, they were very similar across centers. With FFP and lyophilized plasma showing less blood loss compared to hexten, which was the coagul originally recommended by the military. And then we looked at coagulation parameters. In this case, all the samples were sent to one of the centers. Because of variability in lab values done at different labs, we were able to do them, we did them all in one lab and you can see that you get coagulopathy after PT, with hexten. We've also, this is a model where the animals were basically instrumented under anesthesia five days before the study and then they were, this was a conscious hemorrhage where the animal was sedated with

Midazolam and blood about 50% of their blood volume. The small insert graphs show what we did here was we modeled an uncontrolled hemorrhage, but performed the hemorrhage in a controlled fashion. So previous studies, we could get blood loss in real time and then model the way the blood was lost in a controlled setting so that we could look at kind of the effects of an uncontrolled hemorrhage, but, with less variability. And then we were able to resuscitate the animal with various fluids. 24 hours we repaired the injury, gave them the shed blood back and then followed them out for two days to see if they developed any organ failure. And you can see that we get lots of data with lots of different fluids. And the way to sort of normalize all that is to do area under the curve, and as you can see, in this case, on the right side of the lower graph is fresh whole blood. Cardiac indexed was maintained best with whole blood compared in this case we had an HBOC and LR. And the whole blood was one of the better fluids. Plasma lactate, again you follow the

different fluids and you can see whole blood was the best in keeping lactate down. We can also look at oxygen debt and in these swine models, three is about normal in these animals. And what we've observed is that if the oxygen debt falls below one, the animal will not survive through the study. And we see again that whole blood is good. And whole blood is good with less blood volume compared to some of the other fluids. And then we have the survival rates that show, again, whole blood had the highest survival. To do some more uncontrolled hemorrhage models, we've performed a hybrid model, where basically we do an initial controlled hemorrhage and 24 mLs per kilo, and then do a spleen injury, allow the free bleeding for 15 minutes. And the nice thing about doing a splenic injury model is that if we wanted to survive these animals, we could then do a splenectomy and then recover the animal. And see how different resuscitation would improve survival. And in these acute studies, we've gone out six hours. And here is one where we were

trying to add data to the ratios of blood product being used. And we compared whole blood one to one, one to four. And you can see that fresh frozen plasma was good, as well as the blood products, in reducing blood volume and compared to hexten again. And the survival again, this was a low volume resuscitation, so it was a limited resuscitation with the blood products and basically showed that the survival was not quite as good as had seen, where you can repair the animal and give them their blood back. In this situation, it showed that low volume resuscitation with whole blood or ratios about half of the animals survived, suggesting that either they needed another dose, or you need something else. Another model that was done by our colleagues in the UK was where they've inserted a blast injury on top of the hemorrhage, and then looked at resuscitation. And I'm just going to show a quick slide on survival. And this sort of changes the paradigm. But if you do hypotensive resuscitation after a blast injury, and this is using

crystalloids, so it's not quite as relevant to this audience. But just the idea that the right, on the lower graph, the lower bar graph you see that hypotensive resuscitation after a blast injury had the lowest survival. So as we get into concluding my remarks, is that people question about the reproducibility of some of these uncontrolled hemorrhage models. Well, this shows three separate models with different fluids. And you can see that the reproducibility is not so bad. There is variability, but in general, they've all acted similarly to some controlled spleen injury model. And so, some of the kind of conclusions that we've taken from these is that large animal models seem to be well suited for gross evaluations of resuscitation fluids and drugs. But they may not be sensitive enough to see dose response effects. And we base that on kind of our response to the one to one, one to two, one to four blood plasma to red cell ratios. But after the results of the proper trial, maybe this is very similar to how

people respond. But still, trying to see some subtle differences, these models may, or we're not measuring the right endpoint for them. There's a variability in the amount of bleeding from uncontrolled hemorrhage and we can reduce that by using these hybrid models. These large animal models are also applicable to include standard critical care practice, if desired. And the swine can be recovered for survival studies. The poly-trauma models require the animals to be anesthetized, so the goal is to use the anesthesia with the least effect on hemodynamics. The large animal models can be adapted for multicenter studies, as I mentioned. And they are well suited to evaluate hemodynamics, coagulation, indices of inflammation, metabolic responses, including oxygen metabolism and efficacy of red cells. And I would submit that, I think from what I've learned this morning is that clinicians and basic scientists working together with these models, I think we can begin to answer some of the questions that have been raised. And hopefully we can make

progress in improving red cells. So, I guess we'll answer questions part of the panel. Thank you.

(Applause)

DR. SPINELLA: All right, our second speaker in this session is Dr. Sylvain Cardin. Sylvain is the Chief Scientific Director of the Naval Medical Research Unit, also in San Antonio. So, Sylvain.

DR. CARDIN: Good afternoon, everyone. And thank you to the organizer to invite me here to share the result of what we had done in San Antonio. I'm the new Chief Science Director. I joined the group in June, so everything is still new for me. Today I'm presenting data for our trauma surgeon, Forest Sheppard. And it is a model that is in development. What I'm going to do today is I'm going to walk you through what they have been through, and how they have come to develop a model that I hope to convince you it is pretty similar to trauma in human. As Dr. Dubick has mentioned, we are military, so we do aim to develop model where we can study, for instance,

prolonged field care situation. How can we study how to take care of our war fighter in the field in the best similar way if you will. And that's what I hope I will be able to demonstrate. The model that we are working is the non-human primate. Here's the normal disclaimer. There is two kinda population of non-human primate. We have the old world non- human and the new world. The major difference is the region where they come from. But also there is some difference in term of the way they look, they way they act. For instance, the new world monkey uses his tail as a fifth arm. It's much smaller. This one is larger or something. This one is monogamous, this one polygamous. Not that it's going to make a difference. So, the reason we're using the rhesus monkey is as you can see, in terms of differentiate, the apes, which comprise the human, the measure of those species are all endangered, or can not be worked on. So, the closest one is the old world monkey. That includes macaques and baboon. Although we did some experiment in

baboon, it is much more difficult because of the size and all the difficulties that goes with it. The rhesus monkey is smaller, it's easier to take care of, and you will see, as we go, the difference. So in term of homology to the human, in term of protein, the gorilla, as you can see, because it is very close. The closest one is the rhesus monkey, which we're using, which you can see is higher than the other model. In term of immuno acid from skins of the human factor ten and seven, the rhesus monkey is relatively close to the human. The chimpanzee, which is an ape, is almost similar. As you can see, overall, in terms of homology, although those are only some protein, they are relatively similar. This graph here shows that inter-species hematologic chemistry and coagulation comparison between different species. Mainly here what to take is the human and the rhesus monkey have very close, closer than the pig and the cyno-monkey, and here we have put our mammals in a bank of data. Which compares almost 300 non-human primates. And we are very similar to

the human, especially as it similar different methodology, renal function, liver function and coagulation profile in comparison. Therefore, it is good, it is feasible to compare both. Here we have a couple of xenotransfusion considerations, as you can guess, there is a problem in terms of xenotransfusion. There's an exaggeration, aggregate of human platelets when we infuse in the swine. There's a thrombosis complication reported in one study in which human platelets derived from immunostatic agent infused in swine hemorrhagic model. Corsine platelets glycoprotein have been shown to be recognized by human natural and antigal antibody, which create many problems as you can see here. And mainly, there is genogenetic immunological compatible between the rhesus monkey and the human derived blood product, as it has been demonstrated by many studies. Also here, just an internal test. When we put human plasma in recipient in terms of rhesus monkey, swine, and human, there is no reaction for when you infuse it in the rhesus monkey and the human, where there is

a reaction for the swine. Therefore, human plasma and human platelets infusion are compatible in rhesus macaques and we are currently undertaking a study to determine human PRBC compatibility in rhesus macaques. So here, the measure of the non-human primate trauma model in the literature are mainly baboons. And as you can see here, which lead to the latest development that our lab have done. So, now hopefully you can see why the rhesus monkey. It is widely available, the size is relatively good. There is a widespread familiarity in term of using and taking care the rhesus monkey. The real agent are readily available. You can buy rhesus monkey regent. But more importantly, you can use a human reagent for different aspect. It is our old world primate, so very close to human. Although costly, the return on investment is high. So because of the size, because of what you can get from that model, it is a good return on investment, and you'll see a little bit later. So, what are the model that we have developed in the lab. You have the first one

was the uncontrolled liver hemorrhagic shock. PT is a pressure target controlled hemorrhagic shock. Thirty minute, sixty minute. Sixty minute with a soft tissue injury that you will see. And sixty minute plus soft tissue injury and muscular-skeletal injury. I don't want to destroy the punch, but basically these, even though those are very strong models, especially here, the trauma response is not as high as we would have expected. So we had to go to a more complex model, which is a pressured target control hemorrhagic shock. Although in this one, what we have done is we let the animal go to decompensation in addition to a soft tissue injury and muscular skeletal. I'm going to go relatively quick through this one, and spend a little bit more time. So basically what happened is they have increased the model in term of severity. And even with increased severity, the response was not as close to the human as they would have wished. So this is the first paper that they have published. And mainly, I'm not going to go through all those data, but even though with

the 60% hepatic dummy, the response to trauma was not similar to what we see in war fighter. So basically, on this model, what come is, even with 60% removal of the left lobe of the liver, which is a grade four, hemorrhage appeared to stop once a three reached the map of twenty millimeter of mercury. So, as you can see, it come in here and it rebound. The hemorrhage stopped and there's not much that happened after this. So what they have done is, they decide to go and start to do a controlled hemorrhage that stop and response to complex if you will. They decide to do a controlled pressure target. But they will maintain it at 20 millimeter of mercury. For 30 minutes and 60 minutes. Later you will see they will do a 15 centimeter laparatomy, which is the soft tissue injury, and also had the femur fracture, which is a mid-shaft fracture of the femur at five centimeter. This is our model, and in the model, we look at survival after initiation of shock for 24 hours. And this is all the list of reading that we take from these animals. Because they are

bigger, and we have better chance to get the data. Ok, the first one that I've talked to you is the pressure target controlled hemorrhagic shock for 30 and 60 minutes. Basically, cauterized place, the baseline is the issue. There is a hemorrhage that starts by opening the stopcock, let the pressure going to 20 millimeter of mercury. And every time that it bounce back, it is re-opened. And it last for 30 minutes in the 30 minute group, and 60 minutes in the 60 minute group. After this, there is a re-station period in which 30 minute of crystalloid normal saline, 30 minute of 50% shed blood that it is the animal's blood that is re-infused and after 60 minutes of crystalloid. After there's an observation under anesthesia through 260 minutes, and after survival period. But I will not, we will address, but not in detail in this talk. So basically, the 60 minute, as you can guess, have loss more blood. The parameter are decreasing, but not so much very different between the group at the exception of the lactate here. So those data are not really similar to our military

population that come with multiple injury. So the next model, what they have done is placed (inaudible) and after this, create a soft tissue injury, the laparatomy; muscular-skeletal injury, the five centimeter resection; and hemorrhage. And they were really thinking that with this, that should be enough to create a very strong response. Well, in this one, it's the same principal as what I explained for the shock. The re-station is the same. The exception that the re- station, there was a repair of the laparatomy and repair of the femur by putting the plate and stabilizing the fracture. As you can see here, person blood loss, a little higher in the group with soft tissue injury and fracture of femur. But again, either there's an increase or a good decrease in base deficit, the measure of the data is still the same. But not still the same, but similar between the group. But still not perfectly in line with war fighter that we are seeing. So, to capitalize and re-capitalize what we have talked here, the worst case scenario, which was the pressure target

controlled hemorrhage for 60 minutes with soft tissue injury and femur fracture at the 86% survival and moderate physiological, metabolic and coagulation and inflammatory derangement. So, the animal, as the human, is very resilient to this kind of insult. So we were still not there. So, what they have decided to do is, what I mentioned to you, is let the animal go to decompensation and the way they define decompensation is lost of compensatory shock is defined as 25% reduction in the average of mean arterial pressure that is maintained between zero to sixty. If you will remember, it bumped up, they decrease. So they take this average compensatory increase for seconds. So if it come and it cannot bounce back by itself, they will let it bounce back the first time. The second time that it doesn't back bounce, they start, and this is considered the beginning of the end of the shock. So, for instance, if the average between time zero and time 60 was 23 milligram of mercury. A 25 reduction would be 18 millimeter of mercury as a

trigger point. So this is how they would restore them, the re-station process. So, in this one, as you can see, the map is similar, but now we start to have a bigger base deficit. I forgot to tell you that the way that this graph is a little bit misleading, this one is baseline, end of shock, end of re-station, and after the period of kinda recuperation. So the base deficit is lower, now getting a little bit closer to the human. Lactate is getting higher and is different from the worst group that we have seen in the past, which is pressure target 60 minute with soft tissue and femur fracture. If we look at the coagulation pattern, you can see that the coagulation pattern is worse in the group with decompensation. Same thing with the PTT. The fibro chain, they are relatively similar, but they are relatively low. The same thing in the other parameter, decompensated animal is getting closer to what we see in the war fighter. Same thing when we use the war time, which is thromboelastometry, Dr. Dubick presents on. The group that is decompensate is

higher for the exit. I'm not a specialist of all these details, but just believe it that it's a decrease in the coagulation pattern. So basically, we are getting closer to what a military war fighter. In term of aggregation, there's a decrease in aggregation. When simulated challenge by an antagonistic DIDB, which is the one that we see the biggest difference. The other one, although decreased, relatively similar. But again, mimic the new. So, an HP consideration, although those data show you that it's not like perfect model of war fighter, you can see that we are getting there. And today, for this talk, what has been clear are coagulation. But we have data that show that, as you will see, the immunological response is also very close to the human. So, what do you have, what is the consideration of using non- human primate? The phylogeny, other than it, there's no closer to the human than the old world monkey. There's an extremely high protein homology, multiple protein, to include the coagulation factor like we have put in.

Xenocompatiibilty, human product, blood product, into the old world monkey works. The physiology is close to the human. It can bleed spontaneously in supine position. Like the splenic congestion during shock, like the pulmonary lung pooling in response to shock. And the complement activation, not presented here, are very comparable to the human. The coagulation profile, as hopefully I have demonstrated to you, the response to poly-trauma hemorrhagic shock are very similar to observe in human, although not perfect, we are getting there. And the next point is what I just mentioned to you, is how the immunologic response, not here for the talk, the initial reports suggests that the immune response of our non-human primate poly- traumatic model is very similar to human response. Those are positive, but it's not everything that is positive about using non-human primate. The first is that you need to have a facility that can handle non-human primate, the cage, the willing to have some person that are willing to handle non- human primate. The cost,

and the per diem is relatively high. Although I have mentioned to you earlier, there is a good return on investment. There is a lot of occupational health step that you really need to address. You know, TB monitoring, all these. You have to have for animal model, but not as complex as this one. In term of personnel, you need more personnel. You need to always have two people with the non- human primate, one being manipulate. You can never leave the animal left alone while out of the cage. Although, true of the majority of the animal model, it takes experienced veterinarian personnel, which we are very fortunate to have at San Antonio. The animal model costs, I made one, I told you for 2,000, it's the low end. The cost is between 6,500 and 9,000 per animal, and without counting the shipping and quarantine period. Of course, as the other, you still have the institution scrutiny. But when you work on non-human primate, it is even worse. The oversight is stronger, the administrative aspect is higher. The safety is also higher to get taught clear, it

can take more than four months, because non-human primate is very high visibility and you can see how it could cause problems. One of the advantages, you can use human assay kit, reagent, and you can also use pediatric probe. Although not really similar, here they say similar, the non-human primate study cost is close to a true clinical trial study, which is around 25 to 40,000 per animal. Too, this is a little bit on the high side. But it is something to consider when you work with non-human primate. Again, is this model perfect? No. Although we are making very big steps to be very close to our war fighter. And as the months come, you will see publication from us in terms of immunologic modulation substance, that is coming. And we have start working on TBI with this model. So, in addition of having femur fracture, laparatomy, and hemorrhage, we will have TBI. Which ultimately, that would be very, very close our war fighter injury that we see in the field today. Thank you very much for your attention.

(Applause)

DR. SPINELLA: All right, so we are at the last discussion panel for the day. For Dr. Dubick, who is coming up too. So I guess while some of you are thinking about questions to ask, Mike, I'll go ahead and start with the first question. So, in your conclusion slide, you did say that the pig models could be used to measure red cell efficacy. But when it comes to storing red cells, is it possible to store pig red cells in the similar way that human red cells are stored? So that the data would be translatable?

DR. DUBICK: The only evidence that we have is that one day stored pig red cells is equivalent to about a seven day storage in humans. We haven't done any longer term storage studies. And I don't recall seeing any in the literature either.

DR. SPINELLA: ok. Sylvain, for you, with the primate models that you've started so far, you showed us a lot of very interesting data, but no data with human red cells in these non-human primate models. But then you did say

it's possible. So, I guess, you know, what do you know that you didn't show on the slides? How much detail can you give us?

DR. CARDIN: The preliminary data showed that PRBC are compatible in non-human primate, in the rhesus monkey.

DR. SPINELLA: ok

DR. CARDIN: That's the initial

DR. SPINELLA: By compatible, they are significant hematologic reactions, etc?

DR. CARDIN: Yes

DR. SPINELLA: How many have you done so far?

DR. CARDIN: That, I, not that I can not stay. I just don't know the answer. I think close to 50.

DR. SPINELLA: And we've heard a lot today about oxygen delivery metrics, both physically in the mouse or hamster models. Do you think what was presented earlier today would be possible to also measure in the non-human primate models?

DR. CARDIN: Yes. It would be possible. Although it seemed the model that was present was very interesting, so I would want to know a little bit more. But yes, no, it would be feasible.

DR. SPINELLA: Great. Because ultimately, while clearly super expensive, if we could evaluate direct level measures of oxygen delivery, in these models with human red cells, and develop, whether it be in addition to the trauma models, the sepsis models as well as other shock models, or even chronic transfusion potentially, I guess. It might be the ultimate way to develop studies with clinical outcomes that we could then link to the surrogate measures that we heard about in the morning.

DR. CARDIN: Yes and that would be something that I think is feasible. Not only feasible, but we'll be undertaking.

DR. SPINELLA: Dr. MacDonald

DR. MACDONALD: I'm Vic MacDonald, US Army.

DR. SPINELLA: Mike wanted to follow-up,

sorry.

DR. DUBICK: Just want to make a comment regarding that. So, one of the, I guess advantages of the swine model in that it's less costly as the non-human primate, is that we developed a swine blood bank. And so we can look at swine red cells. So if you have several let's say, they're new storage solutions, or other factors regarding the red cell, that you're interested. You could do kinda a balanced selection study in swine to sort of inform which ones you may want to put in a non-human primate model.

DR. SPINELLA: Yes. And although the reply there, is that the hemostatic system of the pig is also very, is not super similar to humans. They seem to be hypercoagulatable. And at least when you get into trauma models. And while they've been used a lot for trauma, there's a lot of concern out there about the use of them. With hemostatic measures.

DR. DUBICK: Certainly it's normal for a pig. And they do have very high platelet counts.

So normal platelet counts can be 600. So we try to normalize to per platelet, or per thousand platelets.

DR. SPINELLA: Gotcha. All right. Vic.

DR. MACONALD: If I remember correctly, swine red cells use inosine natively as their energy source. They don't metabolize glucose very well, if at all. And that might be a slight problem, I don't know what other differences there would be in terms of using it to screen human storage solutions. So you really have to keep that in mind.

DR. DUBICK: That's a good point, cause we store our swine red cells in regular human storage bags. No one has done a study to look at better swine storage solutions. No one wants to pay for that and no one wants to take the time to really develop that. It is a good point

DR. SPINELLA: All right. If there are no other questions, we are adjourned for the day. Nine o'clock tomorrow morning, see you bright and early. Thank you.

(Applause)

(Whereby, at 4:47 p.m. the PROCEEDINGS were adjourned)

* * * * *

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

Notary Public Number 351998