Food and Drug Administration
Center for Biologics Evaluation and Research

59th Meeting of the

Cellular, Tissue, and Gene Therapies Advisory Committee

February 25, 2014

Hilton Hotel
Grand Ballroom
620 Perry Parkway
Gaithersburg, MD

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PROCEEDINGS (8:00 a.m.)

Agenda Item: Call to Order and Opening Remarks

DR. SNYDER: Welcome, everyone, to the 59th meeting of the FDA Cellular, Tissue, and Gene Therapies Advisory Committee. It should be a very enlightening and interesting two days, and today's topic is going to be particularly quite stimulating and interesting.

As you know, the topic will be oocyte modification and assisted reproduction for the prevention of transmission of mitochondrial disease or treatment of infertility, and this will occupy all of today and the first half of tomorrow. Before we get started, if Gale could read the Conflict of Interest Statement, please.

Agenda Item: Conflict of Interest Statement

MS. DAPOLITO: Good morning everyone. The Food and Drug Administration convenes the February 25 and 26 2014 meeting of the Cellular Tissue and Gene Therapies Advisory Committee, under the authority of the Federal Advisory Committee Act of 1972. With the exception of the industry representative, all participants of the committee are special government employees or regular federal employees from other agencies, and are subject to the Federal Conflict of Interest Laws and Regulations.

The following information on the status of this

advisory committee's compliance with federal ethics and conflicts of interest laws, including but not limited to 18 USC Subsection 208, are being provided to participants at this meeting and to the public. FDA determined that all members of this advisory committee are in compliance with federal ethics and conflict of interest laws. Under 18 USC Subsection 208 congress authorized FDA to grant wavers to special government employees and regular government employees who have financial conflicts when it is determined that the agency's need for a particular individual service outweighs his or her potential financial conflict of interest.

Related to the discussions at this meeting, members and consultants of this committee have been screened for potential financial conflicts of interest of their own, as well as those imputed to them, including those of their spouse or minor children, and for the purpose of 18 USC 208 their employers. These interests may include investments, consulting, expert witness testimony, contracts, grants, CRADAs, creators, teaching, speaking, writing, patents, royalties, and primary employment.

This meeting includes three topics. For topic one the committee will discuss oocyte modification and assisted reproduction for the prevention of transmission of mitochondrial disease or treatment of infertility. This is

a particular matter involving specific parties. For topic two, on February 26 2014 the committee will hear updates on guidance documents issued from the Office of Cellular Tissue and Gene Therapies' Center for Biologics Evaluation and Research. This is a non-particular matter. For topic three the committee will discuss considerations for the design of early phase clinical trials of cellular and gene therapy products. This is a particular matter of general applicability.

Based on the agenda and all financial interests reported by members and consultants, no conflict of interest wavers were issued under 18 USC 208. Dr. Jane Lebkowski is serving as the industry representative acting on behalf of all related industry, and is employed by Asturias Biotherapeutics in Menlo Park, California. Industry representatives are not special government employees and do not vote.

With regard to FDA's guest speakers the agency has determined that the information provided is essential. The following information is being made public to allow the audience to objectively evaluate any presentation and/or comments. For topic one, doctors Dieter Egli, Mary Herbert, and Shoukhrat Mitalipov have associations with firms that could be affected by the committee discussions.

There may be regulated industry speakers and

other outside organization speakers making presentations. These speakers may have financial interests associated with their employer and with other regulated firms. The FDA asks in the interest of fairness that they address any current or previous financial involvement with any firm whose product they may wish to comment upon. These individuals were not screened by the FDA for conflicts of interest. This conflict of interest statement will be available for review at the registration table.

We would like to remind members, consultants, and participants that if the discussions involve any other products or firms not already on the agenda for which an FDA participant has a personal or imputed financial interest, the participants need to exclude themselves from such involvement, and their exclusion will be noted for the record.

FDA encourages all other participants to advise the committee of any financial relationships that you may have with any firms, its products, and if known its direct competitors. Regarding media inquiries, the media contact is Jennifer Rodriguez of the FDA Office of Media Affairs. And we also would like to remind you to silence your electronic devices. Thank you.

Agenda Item: Introductions

DR. SNYDER: Thank you. I think what we'll do next

is have everybody sitting around the table, both members and guest members, introduce themselves very briefly, just stating your name, your affiliation, and very briefly your area of expertise.

DR. LEBKOWSKI: Jane Lebkowski. I am with the Asterias Biotherapeutics. I am the industry representative on this committee. My area of expertise is cell and gene therapies.

DR. DIEKEMA: Doug Diekema from the University of Washington and Seattle Children's Hospital. My area of expertise is bioethics.

DR. KEEFE: David Keefe from NYU Medical Center. I am a reproductive endocrinologist and fertility specialist, does ART and studies reproductive aging in the oocyte.

DR. WENSTROM: I am Katharine Wenstrom. I am from Brown University Women and Infants Hospital. I am an OB-GYN and I specialize in maternal fetal medicine and clinical genetics.

DR. STEINBOCK: I am Bonnie Steinbock. I am a professor of philosophy at the University of Albany, with a specialization in bioethics, and particularly reproductive ethics.

DR. MORAES: I am Carlos Moraes. I am a professor in the Department of Neurology, University of

Miami. I work with mitochondrial genetics and mitochondrial disease.

DR. WOODRUFF: My name is Teresa Woodruff. I am reproductive biologist, working in the Department of Obstetrics and Gynecology at the Feinberg School of Medicine in Chicago.

MS. REEDER: Good morning. My name is Sharon Shaw Reed. I was diagnosed with mitochondrial disease 14 years ago. I am honored to serve on the FDA Mitochondrial Patient Representative Committee. Also I have served with the United Mitochondrial Disease Foundation, volunteering on behalf of the mitochondrial community. Thank you.

DR. ROSE: Steve Rose, Chief Research Officer at the Foundation Fighting Blindness, molecular genetics, transplantation and immunobiology.

DR. GEARHART: John Gearhart, University of Pennsylvania, regenerative medicine.

DR. COUTURE: Larry Couture, Senior Vice President for Applied Technology Development, The City of Hope National Medical Center, and cell and gene therapies in general.

DR. LEE: Mei-Lan Ting Lee, professor at the University of Maryland. My expertise if biostatistics and genomic data analysis.

DR. GOLDMAN: Steve Goldman. I am a neurologist

at the University of Rochester. Specialty in stem cell biology and CNS regeneration.

MS. DAPOLITO: Gail Dapolito, Center for Biologics Evaluation and Research, FDA, designated federal officer for the Committee.

DR. SNYDER: Evan Snyder. Sanford-Burnham Medical Research Institute and UCSD. I am a stem cell biologist, also a pediatric neurologist and neonatologist.

DR. AHSAN: Tabassum Ahsan, Tulane University, Department of Biomedical Engineering, stem cell bioengineering and tissue engineering.

DR. DAHLGREN: Linda Dahlgren. I am at the Veterinary School at Virginia Tech. I am a large animal surgeon and I work in the areas of stem cell biology and tissue engineering.

DR. CRIPE: Tim Cripe, Chief of Hematology/Oncology and Bone Marrow Transplant at Nationwide Children's Hospital, affiliated with the Ohio State University. My expertise is in non-catalytic viral therapy for pediatric cancer.

DR. EMENS: Leisha Emens from Johns Hopkins University. I am a cancer immunotherapist with a special based to date on cell based therapies.

DR. BUGBEE: I am William Bugbee, orthopaedic surgeon, Scripps Clinic and UCSD. My area of specialty is

osteochondral transplantation and cell tissue engineering for orthopedics.

DR. CEDARS: Marcelle Cedars. I am a reproductive endocrinologist at UCSF and my areas of interest are oocyte aging and polycystic ovary syndrome.

DR. COHEN: Bruce Cohen. I am professor of pediatrics at Akron Children's Hospital. I am a pediatric neurologist and spend my time in mitochondrial medicine. I am also involved in clinical trials and brain tumors, neurofibromatosis and mitochondrial disease.

DR. PERA: I am Renee Reijo Pera and I am the vice president of research at Montana State University. I just moved there from Stanford University. My research focus is on the generation of oocytes and sperm from stem cells and also understanding human embryo quality.

DR. BUSTILLO: I am Maria Bustillo. I am a clinician. I am at South Florida Institute for Reproductive Medicine. I have been doing egg donation for 30 years. My special interest is assisted reproduction.

DR. SCHNEIDER: Bruce Schneider. I am the Medical Officer in the Office of Cellular Tissue and Gene Therapies and Center of Biologics at FDA.

DR. HURSCH: Deborah Hursh. I am a cell and gene therapy product reviewer with a background in cell and developmental biology a FDA.

DR. WITTEN: Celia Witten, Office Director of Office of Cell Tissue and Gene Therapy.

MS. DAPOLITO: And we also would like to introduce Rosanna Harvey, the Committee Manager and Specialist for the Committee. Thank you.

DR. SNYDER: Thank you very much. I think next Dr. Witten will give us a brief introduction as to what brings us all here this morning.

Agenda Item: Topic I: Oocyte Modification in Assisted Reproduction for the Prevention of Transmission of Mitochondrial Disease or Treatment of Infertility

DR. WITTEN: Thank you Dr. Snyder. I'd like to welcome the members of the Cell, Tissue, and Gene Therapies Advisory Committee, special government employees, invited speakers, and members of the public, and to thank the members of the FDA who aided in preparing for this meeting. Over the next two days this advisory committee will discuss issues related to two separate topics.

The first topic is on potential clinical trials of mitochondrial manipulation technologies to prevent the transmission of mitochondrial disease from affected women to their children, and for the treatment of female infertility. That topic will take place over the next day and a half. Following that discussion we'll hear tomorrow a brief overview of the status of our office's guidance

development efforts, and tomorrow afternoon we will discuss the draft guidance considerations for the design of early phase clinical trials of cellular and gene therapy products, which was issued in July 2013.

The advisory committee's consideration of the draft guidance tomorrow is intended as a separate and independent discussion from the advisory committee discussion of mitochondrial manipulation technologies that's going to take place over the next day and a half. As I mentioned already this portion of the advisory committee meeting is to discuss potential clinical trials of mitochondrial manipulation technologies to prevent the transmission of mitochondrial disease from affected women to their children, and for treatment of female infertility. This portion of the meeting is intended to discuss issues related to the clinical and nonclinical development of the field and not the development of any specific mitochondrial manipulation technology.

This discussion, which is public and invites public comment as part of the FDA commitment to transparency in our consideration of important scientific and regulatory issues. Our primary concern as we cover clinical trials using these technologies is the safety of the women participating in these studies and the safety of any children born to women as a result of participation in

these studies.

The meeting will provide valuable information and perspectives that will help inform FDA deliberations of any clinical trials if such technologies are proposed. This meeting of the advisory committee will focus on general issues and is not intended as discussion of any specific technology. The FDA recognizes their moral, ethical, and social policy issues related to genetic modification of eggs and embryos, and that these issues have the potential to affect regulatory decisions.

However, these issues are outside of the scope of this advisory committee meeting. Accordingly, the advisory committee's discussion will focus on questions of science as well as ethical issues associated specifically with protection of the patients in any clinical trials proposed. We've noted the larger ethical concerns and comments submitted to us to date, and we'll hear more discussion and public concerns in the open session. We'll take this information back to consider whether we need to facilitate a public discussion on these matters, and if so how best to do this.

This morning will begin with Dr. Debra Hursh from the Office of Cellular Tissue and Gene Therapies. Dr. Hersh will provide background and overview of the scientific questions the committee will be discussing. Following her

presentation a series of guest speakers will discuss scientific and research issues. Over the rest of the afternoon and tomorrow morning the committee will discuss the FDA questions on this topic. And now I'd like to turn it over the Dr. Hursh for her presentation.

Agenda Item: FDA Presentation

DR. HURSH: Good morning. We will now begin the scientific portion of this meeting, and I will present some background material before our guest participants speak. Two separate topics will be discussed at this advisory meeting. Today and tomorrow morning the discussion will focus on mitochondrial manipulation technologies in assisted reproduction for the prevention of transmission of mitochondrial disease, or for treatment of infertility.

Tomorrow, after that topic adjourns, an additional topic, a draft guidance for industry, considerations for the design of early phase clinical trials of cellular and gene therapy products will be discussed. This topic is separate from the one under consideration today, and so it will not be discussed further in this presentation.

The Office of Cellular Tissue and Gene Therapies is within the Center for Biologics Evaluation and Research, or CBER, of the Food and Drug Administration. It regulated human cells and tissues, including reproductive tissues,

intended for transfer into human recipients under the Public Health Service Act, section 361. This provides authority for FDA to make and enforce regulations to prevent the introduction, transmission, or spread of communicable diseases.

Section 351 of the public health service act, and the Food, Drug, and Cosmetic Act, give FDA the authority to regulate cell and gene therapy products as biological products and drugs. Under these laws a biological product requires a biologics license prior to its introduction into the marketplace. The FDA regulations specify that such licenses are issued only after a determination that the establishments and biological products meet the prescribed requirements for safety, purity, and potency. While in the development stage biological products may be used in humans if an Investigational New Drug or IND application is in effect.

In 2001 the Center for Biologics issued a letter to sponsors and researchers to inform them that the transfer of genetic material into human cells, in particular gametes, constituted a clinical investigation and required the submission of an IND application to FDA like any other clinical study using manipulated human cells.

This was in response to published reports of the

transfer of egg cytoplasm or ooplasm between an unfertilized donor oocyte and a recipient oocyte. Ooplasm contained mitochondria, thus ooplasm transfer had the potential to alter the mitochondrial composition of eggs. Ooplasm transfer was spreading into clinical practice in the United States, and FDA has concerns about the risks to any children resulting from ooplasm transfer.

CEBR held an advisory committee meeting on the use of ooplasm transfer in assisted reproduction in May of 2002 to discuss what data would be necessary to support clinical studies. The expert committee convened at that meeting determined that there were unresolved safety issues that needed to be addressed prior to allowing further clinical use of ooplasm transfer. Today we will be discussing new methods of manipulating mitochondrial populations that involve transferring nuclear material between eggs and zygotes, or introducing exogenous purified mitochondria into oocytes.

For the purposes of this meeting we use the term mitochondrial manipulation technologies to describe the transfer of nuclear material from one egg or zygote to another to alter the mitochondrial genotype, or the transfer of purified mitochondria from cells into oocytes. I will describe these methods more explicitly in later slides. We will not discuss the transfer of ooplasm

specifically, as we are currently unaware of any intentions to pursue this particular mitochondrial manipulation technology.

The purpose of this meeting is to discuss potential future clinical trials of mitochondrial manipulation technologies, both to prevent transmission of mitochondrial disease from affected women to their children, or for the treatment of female infertility. We will not be focusing on any specific technology. No specific clinical trial, either initiated or in contemplation, is being discussed. And the meeting is not intended to support any specific regulatory action, although the discussions that take place during this meeting may serve to inform potential future regulatory deliberations.

I would like to reiterate the point Dr. Witten made on the scope of the meeting. FDA has specific delegated legal authorities, one of which, relevant to clinical studies, is the protection of human subjects. Many of the mitochondrial manipulation technologies under discussion today may cause heritable genetic modifications to the manipulated oocytes and zygotes, and any children that may be born from studies using manipulated eggs or zygotes. FDA is cognoscente that there are ethical, legal, and social policy issues raised by such heritable genetic

modification of gametes, but these re outside of FDA's delegated authority, and will not be discussed at this meeting.

This meeting is focused on mitochondria, which are subcellular organelles located in the cytoplasm of the cells of higher organisms. The most widely known function of mitochondria is the production of energy in the form of adenosine triphosphate, or ATP, in the process of oxidative phosphorylation. An individual cell has many mitochondria, from hundreds to thousands, depending on its energy needs. However, mitochondria have many other roles, such as production of reactive oxygen species, the regulation of calcium homeostasis, a role in programmed cell death, so that mitochondria are far more than the batteries of the cell.

Mitochondria contain their own genome consistent with the widely accepted theory that they evolved from primitive bacteria. The circular mitochondrial genome is shown on the cartoon on this slide. The mitochondrial genome is small, but because there are many mitochondria in every cell there are many copies of mitochondrial DNA in each cell. Mitochondria are inherited solely from the mother through her eggs.

Due to several factors such as the production of reactive oxygen species, mitochondrial DNA has a higher

mutation rate, much higher than the nuclear DNA that contains the majority of our genes. Mutations in the mitochondrial genome are associated with human disease, many of which are serious and life threatening. Some of the diseases associated with known mitochondrial DNA genes are indicated on the schematic on this slide. Mitochondrial biology and mitochondrial disease will be covered in much more detail by this morning's speakers.

The mitochondrial manipulation technologies under discussion today are assisted reproduction technologies intended to modify the mitochondrial populations of an oocyte or a zygote. Some of them seek to reconstitute the maternal mitochondrial population to prevent transmission of mitochondrial disease from a woman affected by the disease to her children.

However, it has also been suggested in scientific literature that replacement or supplementation of mitochondria in an oocyte may improve fertilization rates in women with infertility. Therefore, both mitochondrial reconstitution and mitochondrial supplementation have been put forward in publications as an assisted reproduction technology that could be used to treat infertility.

There are two forms of mitochondrial reconstitution that have been described in the published literature, and they're schematically shown on these

slides. One is maternal spindle transfer, in which the nuclear spindle of a mature oocyte is removed and placed into another oocyte which has had its spindle removed. The eggs would be subsequently fertilized in vitro.

Another method is pro-nuclear transfer, in which the male and female pro-nuclei are removed from a newly fertilized zygote and placed into a donor zygote in which the resident pro-nuclei have been removed. In both methods the resulting embryo would have the nuclear genome from one woman and the mitochondrial genome from another woman.

A third type of mitochondrial manipulation technology involves the transfer of purified mitochondria from one cell into an oocyte. In this manipulation mitochondria are isolated from specific ovarian cells identified as egg precursor cells, purified and transferred into a mature oocyte at the same time as fertilization in vitro. In this mitochondrial manipulation technology the oocyte of a woman has its mitochondrial population augmented by the introduction of additional mitochondria from other cells of her own ovary. Mitochondrial manipulation technologies will be covered in greater detail by the speakers invited today to address the committee, so I will not describe them further.

As part of the process to guide the discussion for this meeting FDA has developed discussion topics which

can be found in the briefing material that was made available for this meeting. I am not going to read them verbatim. The first topic concerns the availability of animal models or in vitro methods that would be informative of specific safety issues, such as damage to oocytes or embryos, carryover of abnormal mitochondrial DNA, mixing of mitochondrial genotypes, or abnormal embryonic or fetal development.

The second topic concerns the risk to study participants and to children born as a result of participation in any clinical studies of mitochondrial manipulations. The third topic concerns how any first in human trials to assess safety and efficacy of these mitochondrial manipulation technologies might be designed. In particular the FDA is interested if specific pathogenic mitochondrial DNA mutations are most appropriate for first in human trials, or how great the risk of carryover of mutant DNA into the germ line of any female’s offspring is.

In the study of infertility, would assessment of mitochondrial function be of use to screen for enrollment?. What comparators would be used for these trials with the understanding that they would differ for the prevention of mitochondrial disease versus infertility. What types of monitoring would be informative and appropriate for these trials, and what would be the extent of such monitoring.

How would informed consent or assent for these trials be undertaken, and finally what would be the appropriate measurements of efficacy for each indication.

The last topic deals with what controls should exist over the production or manufacturing of oocytes and zygotes by mitochondrial manipulation technologies. In particular, how would the process be monitored for success, and what source controls, such as source of oocytes or other cells, would be advisable. There are exogenous non-clinical reagents and chemicals used in mitochondrial manipulation technologies. How might they be qualified for use in clinical studies? And finally, what genetic tests, if any, might be appropriate for qualifying embryos prior to transfer.

We are grateful to have the participation of a group of highly distinguished speakers. Dr. Jerry Shadel from Yale will present information on mitochondrial biology and genetics. Dr. Salvador Dimauro of Columbia University Medical School will address the topic of mitochondrial disease. Dr. Marc-Andre Sirard from the Université Laval will speak on the biology of eggs and embryos, and Dr. Keith Latham of Michigan State University will speak on existing animal data on nuclear and mitochondrial transfer.

We also have the participation of Dr. Shoukhrat Mitalipov from Oregon Health and Science University, Dr.

Dieter Egli from the New York Stem Cell Foundation, and Dr. Mary Herbert from New Castle University in the United Kingdom to present their own data and experience with the development of mitochondrial manipulation technologies. We thank all these speakers for making the time to participate in this meeting. Finally, I would like to acknowledge all the members of the planning committee for this meeting, without whom it could not have been put together. Thank you for your attention.

DR. SNYDER: Thank you very much. I guess we will just launch right into the educational aspects of the program. Giving a basic introduction to mitochondria and mitochondrial DNA will be Dr. Shadel from Yale University.

Agenda Item: Research and Science Presentations

Agenda Item: A Basic Introduction to Mitochondria and mtDNA

DR. SHADEL: Hello everyone, I am Jerry Shadel from Departments of Pathology and Genetics at Yale University. I guess my charge today is to provide a basic introduction to mitochondria and mitochondrial DNA, and issues with mitochondrial DNA inheritance and stability. First of all, as Deb mentioned in her introductory remarks, mitochondria of course are sub-cellular organelles that are derived from bacteria ultimately two billion years ago or so. They are complicated organelles in the cell, they have

two membranes, and so there are four compartments to deal with. The mitochondrial DNA is actually the furthest inside the matrix of the mitochondria.

Importantly mitochondria are quite complicated. They're comprised of about 1200 to 1500 different proteins, most of those proteins are encoded in the nucleus and imported into the organelle. But the mitochondrial proteome if you will, or the protein repertoire in mitochondria is actually quite tissue specific, so different mitochondria have different protein compositions, functions, and structures within different tissues. So they're tailored really for the specific needs of that particular type of tissue or cell.

They are at the metabolic crossroads, we have already heard that they are important for the production of ATP through the process of oxidative phosphorylation, but they really are at the crossroads of metabolism, there are dozens of other critical biochemical reactions that occur in mitochondria that are essential for cellular function. They are also critical for the process of programmed cell death or apoptosis, particularly the cell intrinsic pathway of apoptosis. I think really an exciting area now I'll talk a little bit more about, because it's probably not thought of by many sort of standard thinking, is mitochondria are involved in signaling as well. They both receive and

generate signals that are important for cellular function, I'll talk a little bit about that.

Finally, we're here because they cause diseases that are maternally inherited, they cause maternally inherited diseases, and they are also involved in aging, so they become mutated and their function declines with age, and so age related pathology is thought to be downstream to mitochondrial function as well.

As I mentioned, mitochondria are sort of always drawn as this double membrane submarine that's cruising around cells, but in reality the mitochondria is actually a very complicated network that's constantly fusing and dividing, and that's depicted here in this slide. Here are two mitochondria that are fusing together to form a larger mitochondria, and in fact they form large elaborate networks that are very dynamic.

One idea of why these mitochondria undergo these rounds of fusion and fission is to maintain the functional integrity of mitochondria. So for example if you divide a mitochondria and one of the mitochondria is nonfunctional, here indicated as a loss of the membrane potential that's required for ATP production, if it doesn't recover it can be targeted for degradation through pathways called autophagy or specific pathways even called mitophagy. So this is really a way to segregate off if you will

dysfunctional mitochondria and get them out of the network and maintain a functional network. If they do regain they can go back into the network and fuse and continue. So one idea is this occurs to maintain a functional network in cells.

Mitochondria are also involved in programed cell death, I just want to give you a real brief summary of that. The breakthrough understanding in that was the discovery that actually one of the electron carriers in the electron transport chain under certain stressful conditions gets released from mitochondria and actually leads to activation of enzymes that promote the process of apoptosis, which involves really a degradation of the nuclear DNA and other cellular features that caused the cell to die and be disposed of in a regulated way.

That original discovery was bolstered by other factors, so there’s actually many factors that are released for mitochondria that have pro-apoptotic roles, and in addition there are families of proteins, the BCL2 family and related proteins, the relative contributions in the outer and inner mitochondrial membranes actually regulate whether a cell will be more or less susceptible to cell death in response to different stresses. So they’re quite intricately involved in the process of cell death.

A really burgeoning area of mitochondrial biology

is the role of mitochondria in the immune system. I think this is best exemplified by the role in antiviral and anti-pathogen responses. This was really spurred by the discovery of an interesting protein called MAVS on the outer mitochondrial membrane, which stands for mitochondrial antiviral signaling.

This protein actually integrates signals that emanate from when a virus infects cells, the nucleic acids are recognized as foreign by cytosolic receptors, and these signals for reasons that are really not clear culminate on the MAVS protein and another protein called STING in the endoplasmic reticulum membrane, and this leads to production of interferon molecules, pro-inflammatory molecules that combat the virus from the cells. So it’s an innate system to combat viral infection in cells, and mitochondria are intricately involved in this, and this is really a huge area right now.

In addition, mitochondria, since they’re derived from bacteria, if they get released due to faulty apoptosis or necrosis or tissue damage, their molecules actually look like bacteria, they look forum, and they can illicit immune responses and inflammation that can contribute to pathology as well. So it’s quite a complicated dynamic here with the immune system and mitochondria.

Finally I alluded to signaling in my introductory

remarks. As I mentioned all of the proteins except for what are encoded by the mitochondrial DNA, which I'll talk about in just a minute, come from the nucleus. The vast majority in fact of proteins that are in mitochondria and control mitochondria are encoded by the nuclear genome that are inherited by both parents, and this really indicates that there must be back and forth signaling between the nucleus and mitochondria to maintain a functional mitochondrial network.

And in the field we sort of define these signals as signals emanating from the nucleus telling the mitochondria what to do or how many to make, et cetera, as anterograde signals, and when the mitochondria are dysfunctional or want to relay signals back to the nucleus to change gene expression to alter their function or recover from stress for example, these so-called retrograde signaling pathways.

And so the field is really actively pursuing what the mechanisms are of these signaling pathways and the proteins involved. As Deb mentioned certainly the most famous function of mitochondria is indeed to make ATP, and the way that occurs is through five large enzymatic complexes that are on the inner mitochondrial membrane.

And what these complexes do is when you eat for example you break down your food into small molecules,

those are oxidized in the mitochondrion to generate NADH and FADH2, which are high energy electron bearing molecules. Those molecules pass electrons down what's called the electron transport chain, and those electronics are sort of stepwise passed down the chain to extract the energy from them in a very controlled way.

And as you go down the chain protons are pumped from the matrix to the inner membrane space, and this creates basically an electrochemical gradient of protons, or a battery if you will, that the ATP synthase, an enzyme that makes ATP, can pump those electrons back, harness that energy, and convert it to ATP. This is an oxidative reaction that usually occurs using oxygen, this is the main reason we breathe as well, most of the oxygen goes to complex four here to be converted to water as the final electron acceptor.

However, electrons can also get passed to oxygen prematurely at different points in the chain to generate what are called these reactive oxygen species, which are these super-oxide ion and hydrogen peroxide for example. These are known to damage other components of the cells. They damage lipids, proteins, DNA in the cell.

And this is really a very well characterized way that mitochondria contribute to pathology, is through oxidative stress, damage to cellular components, and so on.

However, I also want to point out that over the last ten to 15 years reactive oxygen species are also signaling molecules. So there's this interesting dual function of these molecules both as signaling molecules and as damaging molecules that is really being pursued actively by the field.

So with regard to these signaling pathways I just want to point out I think it's important in the context of this meeting to understand that there are ways that cells deal with dysfunctional mitochondria and try to rectify things in real-time.

Here I'm showing a nucleus and a mitochondria, and this is very complicated, but the bottom line is that there are genes in the nucleus that either encode mitochondrial proteins or that regulate the biogenesis or function of mitochondria, and these are controlled by really master regulatory transcription factors in the nucleus that express those genes.

These genes are responsive not only to cellular cues and tissue specific cues for example, but they're also responsive to I guess the overall health of mitochondria if you will. So mitochondria can emanate signals, for example calcium, which we heard about, deficits in energy production that can be read out as ATP and AMP ratios, or as I told you reactive oxygen species can be a signaling

molecule. All of these can relay through various signaling components in the cytoplasm back to the nucleus to regulate those genes that control function to maintain homeostatic. So there really is a beginning to be well understood crosstalk between these organelles that involves these stress signaling pathways.

Not to belabor the point, but the ROS signaling aspect is something that really is becoming quite apparent in the field. And I'm not going to go through all this, but the basic idea is that there are quite a few stimuli that result in the purposeful production if you will of reactive oxygen species. There are sensors that mediate those signals to the nucleus and give you various outcomes in terms of functioning of cells and tissues.

So we really are here to talk about mitochondrial DNA, so I should probably get to that. Mitochondrial DNA in humans is a double stranded circular molecule, it's 16 and a half kilobases, or 16,000 base pairs, it encodes 37 genes, and 13 proteins are essential components of the ox-phos complexes, but all the rest of the ox-phos complex components, around 75 or 80 proteins, come from the nucleus and assemble with those mitochondrial encoded ones to generate these complexes. To express genes in mitochondria you need to do translation, and to do translation you need a ribosome. Mitochondria have their own ribosomes. The

ribosomal RNA genes are encoded my mitochondrial DNA, all the proteins in the ribosomes, again probably 75 or so, are encoded in the nucleus and brought in to assemble with those RNAs. Finally, there are 22 TRNAs encoded by the mitochondrial DNA that work with the ribosomes to express the 13 MRNAs.

This slide simply depicts the relative contribution of the mitochondrial genome in terms of protein components of the ox-phos complexes. Here you can see for example complex one, there are seven components encoded by mitochondrial DNA, approximately 32 by the nucleus. Complex two is actually solely encoded by the nuclear genome. Complex three there's one in ten, three in ten, two in 14, et cetera. So the actual ox-phos complexes are an amalgam of genes encoded by two genomes, except for the so-called complex two.

Mitochondrial DNA is DNA, so mutations occur in it. They occur at a higher rate actually because of the oxidative environment, as Deb mentioned. There are now on the order of 300 or so known pathogenic point mutations, and Dr. Dimauro I'm sure will go into much greater detail on the actual diseases and mutations spectrums and et cetera. But suffice it to say there are multiple point mutations, deletions, rearrangements that are known to be pathogenic, and these can be passed on maternally, through

the maternal lineage, by mom. I'm not going to go into great detail, again, because Dr. Dimauro is going to talk about this. These mutations in aggregate can affect just about every major biological system in the human body, causes major organ dysfunction, blindness, deafness, neuromuscular problems, et cetera.

Let's talk a little bit more about the organization of mitochondrial DNA. First of all, mitochondrial DNA, here shown stained with an anti-DNA antibody, you can see the nuclear DNA quite prominently here, but these speckles or nucleoids they're called in the cytoplasm is actually the mitochondrial DNA in a cell. That mitochondrial DNA is packaged by protein factors, a main component of which is called TFAM, that actually wraps p and packages DNA into these units called nucleoids.

Mitochondrial DNA, as mentioned, is a multi-copy system. So in most cells there are thousands of copies of mitochondrial DNA, and they're packaged into these nucleoids that I just showed you. And this is actually the reason why the genetics are so complicated, and why we're here today talking about how mutations are passed on from mother to daughter. The multi-copy nature of the genome poses quite a few interesting genetic issues that need to be discussed. So first off there's this idea of heteroplasmy and homoplasmy that I'll introduce. There's an

idea that the mutation load, if you will, can drift.

There's a phenomenon called the maternal bottleneck that I'll talk about, there are threshold effects, and then of course all of this leads to very complex genotype-phenotype relationships in mitochondrial diseases, so I'll try to go through some of these in the final moments of my talk here. First of all, there's this idea of heteroplasmy. So again, multiple copies of DNA per cell. And that can lead to a situation where all the copies are the same, or normal, for example. That would be homoplasmy, that is there's a homogeneous population of mitochondrial and DNA molecules, all the same.

However, if you have a mixture of mutated and normal DNA, that is referred to as heteroplasmy. And you can have different degrees of heteroplasmy, a small amount or a large amount. So you can have various loads of mutant mitochondria that represent a large proportion or a small proportion of the total amount of mitochondrial DNA. So that's what those two terms actually mean, heteroplasmy is simply a mixture of two different types or genotypes of mitochondrial DNA. This leads to an interesting phenomenon known as genetic drift in mitochondrial populations. So in a cell that is heteroplasmy, it turns out that during cell division at any stage – embryogenesis, gametogenesis, or even in your normal dividing cells as you age – the

percentage of mutant can shift.

For example, you can go from a cell that has a small amount of mitochondrial mutations to a cell with large amounts over a couple of generations, or even a single cell division, due to the properties of heteroplasmy and the differential replication and segregation of those genomes. This leads to another effect, which is known as the threshold effect. And that is that most mitochondrial mutations don’t have a severe deleterious effect or any deleterious effect until the level of heteroplasmy gets really high.

So even up to 50 percent mutant, half of your mitochondrial genomes are mutates for example or have a disease mutation, they don’t really manifest themselves because the normal ones can make up for that function, or that particular cell type doesn’t have a huge demand for mitochondrial respiration. But once you reach higher levels of heteroplasmy, where you have a large mutation load, this is where you start getting dysfunction in the oxidative phosphorylation system and dysfunction in cells that can lead to pathology.

So this is an unstable situation. You can have heteroplasmy and it can change on you, and that’s a really big deal in mitochondrial disease diagnosis and treatment. A similar phenomenon can happen from one generation to the

next, and that's really why we're here. A mother can be known to have pathogenic mutations but not be symptomatic for example, so have a low load, three percent heteroplasmy, not very much. But because of what's called a maternal bottleneck we can get changes in the frequency in the oocytes from that mother to where they can have none, or they can have a lot of mutated mitochondrial DNA. If this one gets fertilized you obviously have a higher chance of having a child with a mitochondrial disease, whereas these lower numbers you have a less of a chance, and again these can change on you after the child is born.

So what is the nature of this bottleneck, what's going on? We actually don't know. But there are some good guesses. One idea that's out there is that during oogenesis we have a population of mitochondrial DNAs in precursors to the oocytes, these so-called primordial germ cells. And the idea is that the way you go from a low level of heteroplasmy to high or to zero, either way, is that there are random populations or small populations that are chosen for replication and transmission into the oocytes eventually.

So you're not sampling the whole population, somehow you're sampling a smaller population and selectively amplifying those into the oocyte. And that's how we think you can get shifts from the mother to child in

the degree of heteroplasmy pathogenic mutations. There's some sort of bottleneck if you will because of subsets being chosen for replication and/or segregation into the oocyte.

One idea that's come from mouse work is that there is actually a reduction in the total number of mitochondrial DNA molecules in those primordial germ cells. So you go from a lot of molecules down to a small number. That small population is now being amplified back up in the oocyte. So this is one mechanism that has been proposed where you could be bottlenecking. IE, you shrink from a population of thousands and thousands of molecules down to a few hundred, and then those are selectively amplified back up. And depending on the ones that got shrunk down the relative degree of heteroplasmy shifts in what's happening here.

And furthermore, this is actually thought to be a natural mechanism to prevent really deleterious mutations from going on to the next generation. So if you bottleneck down and the oocytes just don't work you can eliminate those oocytes. This is thought to be a way you purify the population of really severe mutations. Furthermore as I told you there are intracellular ways that you might be able to get rid of dysfunctional mitochondria, these so-called autophagy or mitophagy pathways.

So it’s sort of an interesting dynamic here. You bottleneck down, you can select for certain mutations, really bad ones are gotten rid of, but we can still pass on less deleterious mutations which are the ones that actually cause a lot of the human diseases that we say.

This really leads to a logical extension into is there actually normal levels of heteroplasmy. We know about these inherited mitochondrial mutations that cause very stereotypical diseases, but are there natural levels of heteroplasmy in people? And the answer actually is yes. First of all, if you look at human populations, different populations of people have what are called different mitochondrial haplotypes.

So there are point mutations, polymorphisms in mitochondrial DNA that are preferentially in different populations of people. And so we actually all differ from each other at some level on a mitochondrial genotype. Some of these have been linked to either susceptibility or resistance to diseases. So it may be that your actual mitochondrial genotype that you harbor, irrespective of pathogenic mutations, actually has something to do with disease predisposition. In fact these mutations have been used to map migrations of populations from out of Africa into the rest of the world.

Second, as we alluded to mitochondrial DNA exists

in this oxidative environment, these reactive oxygen species can actually damage and mutate mitochondrial DNA. That can lead to dysfunction which leads to more ROs production and even more damage. This is actually sort of the central idea behind the mitochondrial free radical theory of aging, that there's a steady mutagenesis and decline of mitochondrial function that's driven by mitochondrial damage and mutagenesis.

So what does that mean? That means that actually in your cells as you age you are accumulating mutations in your mitochondrial DNA, IE you are heteroplasmic, your cells are heteroplasmic for different types of mitochondrial DNA. Furthermore, the mitochondrial DNA polymerase can make errors when it's replicating the genome, which can also introduce mutations into the DNA. And so the bottom line is that we are heteroplasmic naturally as we age.

Furthermore, there are some data that certain types of mutations can actually clonally expand, i.e. they can increase in number in different tissues, and they're a tissue specific mechanism that frankly we don't understand, but you can get accumulation of specific mutations and specific tissues that can have potential deleterious consequences, for example mitochondrial DNA deletions tend to accumulate in muscles that we don't really understand,

as we age.

A really interesting although maybe a little bit controversial example of homoplasmy is that in many solid tumors there have been reports that there are homoplasmic mutant mitochondrial DNA. That means the whole population shifted over to mutant, and that implies that there may be selection for mutants during tumorigenesis, and that's an interesting example of how you can clonally expand if you will a mutation into taking over the entire population in a cell.

Lastly, there have been several studies on whole genome sequencing of mitochondrial genomes, asking the question are we actually heteroplasmic. Here's a word from Dr. Chinnery's group where they conclude based on their analysis that there are very low levels of heteroplasmy in people, normally. They just took normal people, sequenced different tissues, found that there are different mitochondrial genotypes in different people. And they argue that these are not only due to the oxidative mutations that occur after you are born, but that some of these might be inherited and clonally expand as we age. So I think the bottom line is that we are heteroplasmic naturally, and I think that's important to consider going forward.

So obviously we're here because we're trying to come up with methods to prevent the transmission of

pathogenic mutations from mother to child, and one of the ways that's been proposed to do that is to transfer nuclei from mothers who are affected with mitochondrial mutations into donor oocytes that have been de-nucleated, i.e. getting good mitochondria from the donor and the good nucleus from the mother, and that way you can have a child that doesn't have mitochondrial disease.

The issue I think is is there carryover of mitochondrial sequences even a little bit into the oocyte, what affect could that have. Those are the things I think that you all will be discussing going forward in this meeting.

So with regard to that I think the key points of my talk are the following: First of all, I hope I've convinced you that mitochondria are multi-functional, they're tailored to meet the needs of specific cell types and tissues, and can contribute to disease pathology by quite a number of ways in addition to energy deficits from ATP production.

Mitochondrial DNA is maternally inherited, it's present at thousands of copies per cell, which allows for these natural and inherited heteroplasmic states to exist. The degree of heteroplasmy, i.e. the relative amount of mutant and normal, if you will, can drift dramatically during sematic cell divisions, germ cell development,

embryogenesis, disease states, and with aging.

So all of these things, it's quite a dynamic of heteroplasmy. There's a high threshold for inherited pathogenic empty DNA mutations. That is, you need to get a pretty high level of these before you have a pathogenic outcome. I think that argues that maybe a little bit of carryover into the oocyte by the methods that we're talking about may not be that big of a deal. I mean, if you just only get a little bit it may never amount to anything.

However, I think it remains unclear what's the result of mixing haplotypes in people. If you bring a little bit of DNA in from the donor mom and they're different haplotypes nobody knows what the ramifications of that are. IE, you're bringing in heteroplasmy to begin with. Furthermore, if a few pathogenic mutations go in, how do they interface with the normal heteroplasmy and the other mutations that are preexisting in cells, or that will accumulate with age? So that's all I have to present to you, and I guess I can take questions if that's appropriate.

DR. SNYDER: We are open to questions from the committee.

DR. GEARHART: I have three questions. You said nothing about DNA replication within the mitochondria. Is it constitutive, and could you comment on that?

DR. SHADEL: That is a very important point, actually, and it is thought to be involved in why you can get shifts in heteroplasmy. Normally your nuclear DNA divides, it doubles in a very orchestrated way in each cell division so that you only make the same number of copies each time. Even in non-dividing cells mitochondrial DNA is constantly replicating and being turned over, so there's a relaxed form of replication.

The mechanisms of replication are a little controversial in terms of mechanistic details, but the bottom line is that mitochondrial DNA does replicate all the time, it's turned over and being replicated. And during oogenesis that's thought to contribute perhaps, if there's only a subset of them that are actively replicating a time, or if there are specialized regions within the cell where replication is occurring that could contribute to the bottleneck and the segregation of the mutations.

DR. GEARHART: The second question is if we were to ask of you an issue about quality control in mitochondria, if you would have a set of features or characteristics that you would say this is normal. In stem cell biology, an analogous situation, we're always faced with any cell that we generate in culture, is it really like what's in our body. If you would have a defined N point for mitochondrial function – Now, it's a little bit

complex now, your comment of saying depending on what cell type you're dealing with there may be different things going on within that mitochondria. My question to you would be how do you know it's a good mitochondria for an oocyte, or an early embryo. And if there are, what's the sensitivity of the kinds of analyses you could do to determine that?

DR. SHADEL: I would say there are multiple things you would measure to try to assess the health of the mitochondria in an oocyte, which probably in the end would only be a coarse representation of whether or not they actually are healthy. But I would say obviously the need to maintain a certain membrane potential and be able to do that.

So we have mechanisms to measure membrane potential with dyes, or even mitochondrial function directly with oxygen consumption assays. I don't know how amenable that is to keeping an oocyte out or a stem cell out for very long to do those things. Those are the types of things you'd really want to get at, the mitochondria maintaining a potential, are they capable of respiring as their supposed to.

There are pretty sophisticated ways of measuring reactive oxygen species that you might want to take stock of if they're producing more than normal that that might

indicate that their chain is dysfunctional in some way, that you might want to not move forward if there was too many ROS being produced. But then that becomes a question of we know these molecules are also important for normal cell function, so what is the value you would arrive at that was normal and not too high?

Other than that I think looking at oxidative damage or vicarious mechanisms, looking at protein oxidative damage or mitochondrial DNA. And then of course I think the sequencing technologies we have these days are remarkable. I think you'd essentially want to sequence the genomes and get a handle on the degree of heteroplasmy you're starting with, knowing that that could have a deleterious outcome later. So those are the types of things I can think of off the top of my head.

DR. KEEFE: You mentioned mitochondrial DNA mutations have been implicated in fertility. The irony here though is that embryos are functional anaerobes, they're using the Warburg effect, this anaerobic glycolysis. Are there examples in other settings in which mitochondrial DNA mutations have an impact even in the absence of oxidative phosphorylation, or oxygen consumption? Do they have ox-phos independent effects?

DR. SHADEL: Certainly, for example, if you are under a hypoxic or even pseudo hypoxic situation as you're

referring to, the Warburg effect if you will, that actually leads to the production of more reactive oxygen species. And so one idea is that those reactive oxygen species are going to actually activate what's called hypoxia inducible factor. And so yes, I think there are ways that mitochondrial mutations, even in the absence of respiration, will generate signals that will impact cell biology. That's actually thought to by a component of how tumorigenesis occurs. It's not just the reduction of respiration, it's also signaling of ROS to these other pro-tumorigenic pathways. So I think yes, absolutely the case.

DR. BUGBEE: You mentioned a lot about normal function and disease. Is there any measure of supernormal function, or any way to manipulate that, or can you measure a super-normally functioning mitochondria?

DR. SHADEL: I think that would depend on what the function is that you're trying to augment.

DR. BUGBEE: I wonder if someone has defined what that is and if someone has tried to manipulate it to make more ATP or do something beyond what we call normal, but not necessarily disease related.

DR. SHADEL: I don't know if there is anything that speaks to that. One way that you can augment, Carlos Moraes who was here has shown this actually. One way to overcome dysfunctional mitochondria is actually just to

make more. And so one augmentation that may be beneficial is to make more mitochondria and overcome an energetic or other type of threshold that was being manifested in cells. So there are ways to augment the number. In terms of specifically augmenting function I imagine you could say an antioxidant therapy would be an augmentation, if you're producing way too many ROS you might be able to reduce that and have a positive effect. But in terms of super-mitochondria I guess I don't have any other insights into that.

DR. GOLDMAN: So following that up, presumably the differential expansion of even mutant genotypes in the heteroplasmic mutants reflects some competitive advantage on the part of the mutant genotype. And so is there a commonality to that competitive advantage or its nature across mutant phenotypes that favors core selection of that mutant?

DR. SHADEL: The only really sort of easy to digest example of that would be deletions, where people have proposed that since the molecule is smaller than it might replicate faster. I doubt that's really the case, although that has been proposed as a mechanism. In terms of a point mutation in a TRNA gene for example in imparting a select advantage, I think now you're getting into the previous question of are there certain circumstances like

hypoxia where there's less stringency placed on mitochondrial function where those can have a selective advantage because of other signaling properties I think is an interesting area, but I can't think of any real examples of other types of selective advantages that a specific mutation would impart in growth, other than signaling. I think you can signal pro-myogenic signaling or segregation signals that could be impacted.

DR. COUTURE: So in term of trying to assess the risk of heteroplasmy, is it fair to say based on the notion I guess implicit in the theory of threshold effect, are all of these mutations recessive mutations, or are there any dominant mutations that exist?

DR. SHADEL: I think Dr. DiMauro could help me out here.

DR. DIMAURO: One example, is in the literature, but most mitochondrial DNA you could envision, although the terminology of course is not appropriate here, but mitochondrial DNA mutations are in a sense recessive, because to become pathogenic you have to have a high level, the mutation load has to be high and steep. So in that sense you can consider them recessive.

DR. CEDARS: I just want to follow up on the issues of assessing mitochondrial health, and I think particularly in terms of what we're discussing here. Are

there techniques to do so, to go through some of those areas of mitochondrial health that you can do in the living cell, not damage the living cell, and that represent the total extent of that cell given the variable distribution of the mitochondria in the cell, and perhaps variable distribution of the heteroplasmy as well. So if we sort of have in hand a cell or an oocyte or an embryo that we're trying to decide is it safe, are there ways to do those assessments of mitochondrial health that doesn't damage capacity?

DR. SHADEL: I guess the question is are the standard things that we do amenable to oocytes is the real question. There are dyes that you can add to cells, live cells, that presumably wouldn't have a long-term effect, is something that we could test to measure membrane potential to illuminate the network so that you could visualize it in a cell, make sure it's not fragmented, that it looks morphologically normal. There are fluorescent dyes to detect reactive oxygen species, et cetera.

So the question is, and I don't know the answer, is if you add those to an oocyte do they have a downstream deleterious effect or will they just be cleared and all things will be normal from that point on. I don't know the answer to that, but I imagine that you could come up with ones that met that criterion. But off the top of my head I

don’t work on oocytes, so I don’t know how fragile they are and what mechanisms they have to clear damaged mitochondria, et cetera.

DR. PERA: I just have a quick question on maternal inheritance. I was wondered if you know of any data of how maternal inhereitcen occurs, whether or not there is some paternal carryover or sematic cell carryover. So how is it --

DR. SHADEL: Why is it totally maternally inherited? There are two reasons. First of all, it’s a numbers game. So even if a little bit of paternal DNA gets in oocytes have hundreds of thousands of copies of mitochondrial DNA, so they’re diluted out. But there are actually active mechanisms to get rid of the paternal DNA that gets in. There are ubiquitations of pathways that target the paternal DNA for degradation so that doesn’t contribute into the next generation.

And the way that was shown is that the only way you can really get, in mammals anyway, paternal inheritance, is if you do crosses between species, like mice of different species, not closely related species. You’ll get some paternal inheritance of the DNA because you’ve broken down these targeting and degradation pathways for mitochondrial DNA. So there’s active mechanisms to get rid of it.

DR. CRIPE: Is there ever exchange of mitochondria between tissues as cells? Do mitochondria exist in exosomes, for example?

DR. SHADEL: There are reports of mitochondria traveling through connections in between cells. This occurs actually in the germ line. There's a germ line cluster where mitochondria I believe can be transferred in between cells through filopodia-like structures. There are a couple of other reports of that occurring in non-germ line cell types. So there is indeed evidence for transfer between cells.

DR. SNYDER: Any other questions from any committee members?

DR. MORAES: Not a question, but I just want to continue this discussion of how to analyze mitochondrial function in an oocyte, because I think it is a very important one. I think we really don't know how to do that. We could measure membrane potential I think is the only thing we can measure without killing a cell. But that's not very sensitive. The mitochondria might not be very bad, not very good. So we don't have such a technique, and as Jerry said we don't know if these dyes might damage also the mitochondria. That's a major problem that we have in this area.

DR. SNYDER: Thank you for making that point, and

that will obviously be a topic of broader discussion this afternoon. I think we can now move on to Dr. DiMauro, who is going to discuss mitochondrial DNA related diseases.

Agenda Item: mtDNA-related Diseases

DR. DIMAURO: Good morning, thank you for inviting me. Two apologies. I apologize for I have a nasty laryngitis, my voice may wane on and off. And secondly, I apologize for some repetition that will be in my talk compared to the previous two, but I hope that will not be too much of a problem.

As you all know, as you heard, where do mitochondria come from? About two billion years ago, as you heard, eukaryotic cells were invaded by bacteria, which were adapting better to an increasingly oxygen rich atmosphere, and this symbiance became permanent, and these are the symbiotes that we now call mitochondria. Of course, the bacteria in invading the eukaryotic cells brought in their DNA, and that is why of course all eukaryotic cells, including ours, have two types of DNA, their original tautomers if you want, nuclear DNA, and the mitochondrial DNA, which is a relic but not a genetic fossil.

So what do mitochondria do? You heard that very eloquently, they do very many important things. But arguably one of the most important functions is producing energy as ATP. In this over-schematic representation of

mitochondrial metabolism, I am showing the fuels, carbohydrates and fatty acids, that converge to acetyl coenzyme A, the Krebs cycle.

And I have highlighted the respiratory chain for two good reasons. One is of course the respiratory change as you heard is the business end of mitochondrial metabolism where ATP is produced, and the second reason is that it is the only pathway in the cell where the two DNAs have to intervene, not just nuclear, but mitochondrial DNA as well.

And this is a closed review of the mitochondrial DNA. An exceedingly small molecule, as you just heard, only 16.5 kilobases compared to the three billion base pairs of nuclear DNA, but an important one. And here I have color-coded the 13 genes as you heard that produce, the 13 subunits of the respiratory chain, so that you can see how seven genes encode the seven mitochondria encoded subunits of complex one, there are none in complex two, one in complex three, three in complex four, and two in complex five. Notice the light blue. As you heard from Dr. Shadel the vast majority of the proteins in the respiratory chain are however encoded by nuclear DNA.

Now, although mitochondrial DNA was discovered in the 30s it took 26 years before deleterious mutations, pathogenic mutations in mitochondrial DNA were discovered.

That happened in 1988, when the late Anita Harding in London reported single large-scale deletions in mitochondrial DNA as causing myopathy in a paper in nature, and Doug Wallace reported at Emory the first point mutation in mitochondria DNA causing Leber's optic neuropathy, I'll come back to that.

That had a tremendous ripple effect, of course, and here we are 26 years later with a veritable Pandora's box of diseases in the small mitochondrial DNA which has become crowded with pathogenic mutations of two types: ones affecting protein coding genes, and I show them in red here, and the others, the majority, affecting genes involved with mitochondria protein synthesis, mostly TRNA genes, and I've shown them in blue. In these little boxes I have represented the disorders associated with each mutation, and the variety, the heterogeneity of these disorders is simply astounding, as I'll come back to.

Now, you've heard it before, and it's elementary, but I think it's important in this context to remind you that of course you have a different DNA, you have a different type of genetics. We are no dealing here with mitochondrial DNA related diseases with Mendelian genetics, but with mitochondria genetics. And it differs from Mendelian genetics in three major ways. You already heard maternal inheritance, first of all. Of course we get the

same amount of nuclear information from dad and mom, but all mitochondrial DNA comes from the mother, and only their daughters transmit them to their progeny.

Second, a very important concept, heteroplasmy and threshold effect. Because we are dealing with thousands, as you heard, of mitochondria in each cell, and even more copies from mitochondrial DNA, mitochondria genetics in a very real sense is population genetics, not Mendelian genetics. So when you have a mutation in mitochondrial DNA, usually, not always but usually, not all copies of mitochondrial DNA are mutant, only some of them. So this is a situation called heteroplasmy.

And related to it is a threshold effect. It becomes intuitive that whether or not a certain disease will manifest will depend not just on the presence of absence of a deleterious mutation, but of its number, its relative percent of heteroplasmy, and this is called the threshold effect. This is in itself a relative concept, because of course tissues that are highly dependent on oxidating metabolism will have lower threshold than more glycolytic tissues.

And finally mitotic segregation, you also heard about it, namely that from one generation of cells to the next the percentage of mitochondrial DNA mutants may vary. And therefore not only the genotype, but the phenotype of

the mitochondrial DNA related disorder may change with time. It is not just an abstract concept, it is something that we've verified in clinical practice.

In this slide I represented five exemplary mutations. Two mutations represent two large-scale deletions in mitochondrial DNA causing either Kearns-Sayre syndrome or Pierson syndrome. Two mutations in TRNA genes causing either MERRF or MALUS. I'll come back to the acronyms, don't worry. And one mutation in a protein coding gene, namely the TPA6, calling either NARP or MILS.

But there are only two points I really wanted to make with this slide. The first is to remind you that mitochondrial DNA, as you already heard, is ubiquitous, and therefore as you see here all tissues can, and indeed in many cases are effected, more or less, and so these disorders are very often systemic. And the second point is that despite the morass of clinical presentation there are in fact specific syndromes that we see and that we can rather easily recognize, even at the clinical level.

This again you've already seen. Again, a reiteration, mitochondrial DNA is ubiquitous, so all tissues are affected, but again a reminder that tissues that are more dependent on oxidative phosphorylation, like muscle, brain, heart, are more affected. That's why we often traditionally talk about mitochondrial

encephalomyopathies.

And this give you an idea, this multiplicity, the heterogeneity of clinical representation, of the problem facing the clinician very often when seeing a patient with suspected mitochondrial diseases. I think these disorders are sometimes underdiagnosed when the attitude is this disease is so difficult. What is this difficult disease? And maybe over diagnosed more commonly when the attitude is this disorder is so complex it must be mitochondria. And you have no idea how many complex patients we see that are clearly not mitochondria but are sent to us thinking they are.

So let me now give you a little gallery of mitochondria DNA related disorders so you have a feel for what this field is like. First, single deletions of mitochondrial DNA, the ones described first by the late Anita Harding. Here I represent six different large-scale deletions, as you see, two are identical, in seven patients with Kearns-Sayre syndrome. Single deletions essentially cause three phenotypes, no more.

Kearns-Sayre syndrome, a devastating multisystem disorder of young adults, or its benign counterpart, chronic progressive external ophthalmoplegia, a pure myopathy dominated by ocular among motility problems with ophthalmoplegia, or a disorder that in fact is an

hematological disorder of infancy, Pierson syndrome. These disorders, by the way, and I'll come back to that in a moment, are usually not transmitted maternally. They're not transmitted. So they're usually sporadic conditions. It's very rare that any of these disorders are maternally transmitted.

Let me give you an example, MELAS. Start with MELAS because it's probably one of the most common, if not the most common TRNA mutation, and it is a devastating disorder featuring in typical cases recurrent strokes in children or very young adults, and resulting in severe damage to these children and early death. MELAS stands for Mitochondrial Encephalomyopathy Lactic Acidosis, nothing specific so far, and Stroke-like episodes. The strokes usually affect in the posterior lobes, the occipital lobe and temporal lobes, as you see here. And lactic acidosis can be demonstrated of course chemically, but it can be also demonstrated very well by magnetic resonance spectroscopy. As you see here the presence of these lactic acid double peaks, both in the ventricles, that is in the CSF, and in the parenchyma. Here is a typical affected child, but here is an asymptomatic mother who still shows small peaks of lactate, which are absent of course in normal individuals.

Now let me give you a personal experience that I

think is very appropriate for this meeting. I'm showing you the genotype-phenotype correlation in the first family with MELAS that I started with my colleague Darrel DeVivo at Columbia. This is a family we saw, this is the phenotype.

This is the little girl Victoria that we followed, who unfortunately died at 12 after a series of strokes. Tragic story. She and her family, her mother was normal, was really healthy, and so was her grandmother. There was a brother who was slightly mentally retarded, and that was attributed to perinatal anoxia, but we thought it was much more likely it was due to low levels of mutation, as you'll see in a moment. There was an uncle that was also neurologically affected but we could not see.

But when we started, when we found the mutation, the typical MELAS mutation, the 3243 mutation in TRNA lysine, and we evaluated the percentage in blood, then things became clear, you see that they affected girls at an extremely high percentage of the mutation, but they have unaffected siblings at lower levels. And this sister of the patient, this is a true story, came to Dr. DeVivo and me two years later saying that she was getting married, and she was terrified of having a child like her sister. Could we do anything about it? And unfortunately as I'll show you in a moment perinatal diagnosis for mitochondrial DNA related diseases, and particularly TRNA mutations, is not

possible, so it could not happen.

Another rather common mitochondria-related disease is MERRF, Myoclonic Epilepsy with Ragged Red Fibers. These patients have a number of problems, myoclonus epilepsy, severe ataxia, and ragged red fibers that are shown here, the famous ragged red fibers reported by Ken Gangel back in the ‘60s. This is a cross-section of the muscle biopsy, these are simply fibers where mitochondria proliferate excessively, probably in a futile compensatory attempt, and they are shown by the gomori trichome as reddish patches.

So far I’ve shown you diseases related to TRNA mutations. I’m now showing you a few disorders due to mutation in protein coding genes. The first is the one I already mentioned to you, a double disease if you want, NARP and MERRF. These are due to the same mutation in APA6. Why are two different presentations? For very good reasons. It’s a question of mutation load. When the mutation load is about 70 percent you have young adults showing peripheral neuropathy ataxia, pigmented retinopathy you see illustrated here, you can see it there paternally but of course maternally inherited. But in the same family you may have a baby who is affected by Leigh’s syndrome, a devastating neurodegenerative disease of infants and children, and I’ll come back to this. These children

usually have about 90 percent of the NARP mutation.

Let’s come to Leigh’s syndrome itself. Leigh’s syndrome is, as I already told you, a devastating disorder reflecting the ravages of energy insufficiency on the developing brain. There are symmetrical lesions all along the central nervous system and the spinal cord, but particularly evident at the level of basal ganglia. Leigh’s syndrome is always a mitochondrial disease, although to be sure not always a mitochondrial DNA related disorder. There are many forms of Leigh’s syndrome due to mutations in nuclear genes.

But this is an example of, as I showed you, maternally inherited Leigh’s syndrome. First of all, it’s a tragic disorder because it’s characterized by developmental regression. So these families have a normal child and then they see the child losing acquired skills, stopping smiling, not tracking, it’s a horrible disease, and usually death occurs in childhood. But not always so. This is an example of a typical case of Leigh’s syndrome with profound weakness, really an example of a floppy child.

But it’s not always like this. Occasionally, as in this case, and I had permission from the mother to show this picture, this Italian family, there is a 21 year old girl who has suffered from Leigh’s syndrome since childhood, and this is due to mutation in a complex one

gene, ND3 mutation, and she had an extremely high level of mutants.

The mother also carries the mutation of course, but at low levels, and she's completely normal. There is a slightly younger sister whom I've met, she was a graduate at NYU, and she is completely healthy, and again she is now planning or she is in a reproductive age. No comment, this is why you're here.

Another disorder due to protein codeine gene mutation, Leber's hereditary optic neuropathy, in fact the first point mutation as I told you discovered by Doug Wallace in an American family. This is a disorder of usually young adults, predominantly men, which doesn't fit with the classic mitochondrial genetics. It's almost a tissue specific disorder. By far the most vulnerable tissue are the retinal ganglion cells, or the papillomacular bundle.

So the first symptom that these patients have before developing often complete blindness is central scotoma as you see here, but then very often this kind of blindness enlarges. There are also really pertinent tissue specific mitochondrial DNA related disorders, and we have studied a number of samples of patients who have pure myopathy due to mutations in either complex one, complex three, or complex four, and they show essentially

exercising dollars, sometimes with recurrent muscle breakdown and micro granuloma.

These patients are difficult to diagnose because they often escape the rules for mitochondrial genetics. They're not maternally inherited, they are de novo mutations, and they are tissue specific mutations because the only affected cells are the progenitors of miotes. So do we know everything we need to know about mitochondria DNA related disorders after so many years? Not by a long stretch. There are many unanswered questions. Here I listed three: pathogenesis, prenatal diagnosis, and therapy.

Pathogenesis, simply put, why is Kearns-Sayre different from MERF and MERF from MELAS? After all, all three disorders, as I told you, are due to defects in genes, or essentially to a defect in mitochondria protein synthesis and ATP production. You'd expect a swarm of overlapping symptoms, and on the contrary you have usually rather distinct, there is some overlap of course in practice, but by and large you look at separate distinct syndromes. And the reason why this is we still do not understand really.

Prenatal diagnosis. This is pretty dismal, because for single mitochondrial DNA deletions, as I alluded to before, these are almost always sporadic. A nice multi-centric study conducted by Patrick Chinnery back in

2004 has shown that the risk for a mother to transmit a single deletion to a child is one in 24 births.

Mutation in TRNA genes is a real problem, as I mentioned to you, talking about MELAS, because these mutations are distributed stochastically in different tissues, and the mutation load drifts, as you heard from Dr. Shadel, in time, so that you cannot through the traditional methods of amniocentesis or chorionic villus analysis predict if the fetus will be affected or not.

Mutation in protein coding gene fell slightly different, like the TPA6 that I showed you, because they are distributed more uniformly in tissues. I shouldn’t say uniformly, but more uniformly, and they do not seem to drift as much. So prenatal diagnosis has been attempted in some of these cases, but I frankly don’t think that we can be sure about that.

As you already heard, when human beings moved out of Africa about 150,000 years ago and migrated over the rest of the world, because mitochondrial DNA is prone to mutations, several mutations accumulated and got fixed, and that’s why we have now so-called haplotypes characteristic of different ethnic groups. This is also represented here on the background of mitochondrial DNA, same concept.

And as you heard already from Dr. Shadel it is shown that some of these genotypes either make you

vulnerable to certain diseases or protect you from certain diseases, apparently. But a clear functional role for haplotypes has not been conclusively documented in my opinion. Now, one question that will come up in this meeting, talking about preventing mitochondria DNA related diseases, is is there incompatibility between mitochondrial DNA and nuclear DNA, could there be incompatibility between some of these haplotypes and different nuclear genes.

Well, I think in human beings we shouldn't worry. And I choose to show these two beautiful children from the recent issue of the National Geographic Magazine called the changing faces of America, showing not just these two children but adults too, with the most incredible multi-ethnic mixture, and all looking perfectly normal.

So there are many studies, and this will come up again, about this compatibility between nuclear and mitochondrial genome. In humans we don't have enough data, in monkeys there is some experience, as you hear, and it seems that there is no problem. In mice and fruit flies there are some data suggesting that there may be some problems. And I'd like to end by showing a slide from Carlos Moraes, my former collaborator who is here, and he can comment himself later on that. But I think the ultimate exploration of this incompatibility would be using xeno-mitochondrial cybrids, as he has done, mainly a human

nucleus and mitochondria from apes, or monkeys, from primates.

And Carlos I think will confirm or not, but found minimal incompatibility, if any, when you use mitochondria from chimps or gorilla. It becomes more problematic when you go further away in evolution. I have not mentioned the frequency of mitochondria diseases. Nobody has, actually, mitochondria DNA related disorders. While they're to be sure not common, but they're certainly not very rare. The prevalence was calculated to be one in 10,000 adults, but when you include children I think it's more one in 5,000 approximately.

And very importantly, and Dr. Shadel alluded to that, a study again by Dr. Chinnery, a very interesting study, examining thousands of cord blood from normal children in Newcastle, found surprisingly that one in 200 of these children in fact harbored pathogenic mitochondrial DNA mutations, obviously at very low levels.

But one in 200. You don't see of course one in 200 mitochondria DNA related diseases. But the heteroplasmy is there, and that has also been shown, as you heard from Dr. Shadel, more recently with next generation sequencing at very low level, level of heteroplasmy exists probably in all of us. So I come to my last slide. Are mitochondria DNA related diseases preventable? And this of course is the

subject of the discussion in this panel. Thank you.

DR. SNYDER: We are open to questions.

DR. GEARHART: Just to emphasize the point you made, currently state of the art, if there are any mutated mitochondrial DNAs in the procedure that are done, carried over, we cannot detect this basically until we have an individual that's of age to begin to perhaps demonstrate some of the features of the pathology you've presented. Does that make sense?

DR. DIMAURO: Some present in infancy.

DR. GEARHART: I know that, but there is nothing we can do in a prenatal sense at any point along there.

DR. KEEFE: Of course, they are preventable using donor ague. The cost of course is giving up 0.1 percent of the nuclear genome. That's the difference between a random donor and recipient. Currently you must counsel women that are carrying mutations. What is the percent homoplasmy, for example, of the 243 mutation, as you mentioned the most prevalent, the most devastating? At what point would you recommend that she consider donor egg? This might provide a helpful baseline if we go ahead and start discussing first in human experiments. At what point of heteroplasmia, what percent of the 243 mutation in a woman would you then say have you considered donor egg?

DR. DIMAURO: I don't propose that. I assure you

these women come to us asking is it ready, and I keep telling them no, it's being studied in the United States and in the UK, and hopefully this will be available to you in the near future.

DR. KEEFE: What I asked was a little bit different in starting to think about engineering. By giving up 0.1 percent of her nuclear genome, by using a donor egg, she could prevent this. I think oftentimes when recipients hear the different between her genome and a donor's genome is about 300,000 base pairs, less than 0.1 percent of her genome, do you counsel women affects by MELAS currently, do you counsel them about the possibility of donor egg, using an egg donor?

DR. DIMAURO: They know about there is a resistance there, which is the desire of having their own biological --

DR. KEEFE: Or having 99.99 percent instead of 99.9 percent of a genome.

DR. CEDARS: So one of the things that, if we're talking about a therapy you always want to compare it to what happens without that therapy. So you're saying an affected woman has a one in 24 chance of having an affected child with a deletion. So I would assume, because of the bottleneck issue and because of the heteroplasmy and the variable content, that even if she has had one affected

child, that would not increase her risk over that random rate of having another affected child. I think it becomes very complex, because it’s not as though we’re saying if you carry this your child will be affected, so therefore our therapy that avoids that may have some risk but gives you this large benefit, because she also has a very large chance of having a child that’s affected.

DR. DIMAURO: For a single deletion, that’s yes. But for TRNA mutation, no.

DR. CEDARS: So explain the heredity of those, what’s the risk of transmission with a pathologic phenotype.

DR. DIMAURO: For single deletion, for some reason, and there are some hypotheses about that, the mutation is essentially selected out, and very rarely is that deletion in the egg of the mother, and therefore a child is at risk. But for TRNA mutations essentially all – The mother is a carrier, and all children are going to carry the mutation. At what level, we don’t know. And you cannot judge the level in the fetus in different tissues of the fetus by measuring it in colonic villi or in immunocytes.

DR. CEDARS: But there is no way to estimate, like in that one pedigree you showed us there were two children that had high levels and two that had absence of phenotype.

But there still is some chance of having a normal healthy child.

DR. DIMAURO: Yes, just like the mother in that family, or the grandmother, they could have normal children.

DR. CEDARS: So you could potentially argue what we need are better diagnostic early on potentially rather than therapeutics.

DR. DIMAURO: If that were possible, yes.

DR. MORAES: You mentioned that the prenatal is really hard. Is there no correlation whatsoever with the level of mutations? I know it can drift later on, but if you have cryonic villi that's low in mutation, is there a correlation that the child will have low, or not at all?

DR. DIMAURO: As I understand it Carlos, not for TRNA mutations. For protein coding genes it's much closer. I've seen children, or mothers of children with maternally inherited Leigh's syndrome having their amniocytes studies, and if the level of mutation is very low they've gone ahead with the pregnancy and they've had usually normal children, and vice versa. The curious thing there is that usually in the amniocytes you see either very low level of mutation or very high. Fortunately for us, not that I'm very involved in that, it's very rarely done, but very rarely there is an in between level of mutants which will put you in a very

difficult spot.

DR. SNYDER: Are there any other questions or comments?

DR. COUTURE: So I am sure you don’t see them as patients, but has there been any correlation between mitochondrial mutations or heritable mitochondrial mutations and desirable characteristics, like performance, endurance, strength, et cetera? Can you conceive of a scenario where patients may actually want something other than normal?

DR. DIMAURO: Unfortunately not. And the reason for that is, as I said before, tissues with high oxidative demands like the brain, heart, and muscle, are almost always affected. So these children are usually neurologically involved.

DR. COUTURE: No I am talking about there is something on the order of 300 mutations that have been characterized, and not all have been associated with pathology, is that correct? So we’re talking about a lot of mutations that have not yet been studied in terms of relationship to endurance --

DR. DIMAURO: I can only use an example which however I don’t think has been totally proven, but my friend Doug Wallace, he suggests that some haplotypes confer to mitochondria a tiny bit of loose coupling of

respiration, oxidation, and phosphorylation, and therefore some of the energy is used for heat rather than ATP production, and this would favor survival in harsh climates, like the north pole. You can consider that a posit, but it has not been proven.

DR. KEEFE: There is a Holstein cow that has the haplotype that increases milk production, so that would be one example.

DR. GEARHART: I would add to that answer, I thought that with some of Doug's work too that there was a correlation between military performance in the mountains and these supposedly elements of the Chinese army that do very well at that, and many of these have a mitochondrial mutation.

DR. DIMAURO: Yes, that is a good example, I forgot.

DR. SHADEL: I just want to add that there are mutations that have been associated with longevity. The mutations in centenarians have been mapped that increase life span.

DR. SNYDER: I think we will take a 15 minute break, and everybody should try to be back here by 10:05 please.

MS. DAPOLITO: The committee is reminded that they should not talk about the topic outside of the meeting

room. Thank you.

(Break)

DR. SNYDER: So our next presentation will be by Dr. Sirard, and the topic will be oocyte quality.

Agenda Item: Oocyte Biology

DR. SIRARD: Good morning. I am Marc-Andre Sirard, I am a veterinarian specializing in reproduction, and I'm coming from the French part of Canada, so that's why I have a funny accent. My task today is to introduce the concept of oocyte quality with the perspective of changing the mitochondria content of the oocytes. I would like to stress that in our world mitochondria is not a main issue, it's a side issue in terms of oocyte quality.

So I'm going to spend most of my time explaining what oocyte quality means. I'm going to divide my 25 minutes into a few background information about oogenesis, what makes a good egg, because that's a big issue for infertility patients, and then just briefly review again the building of the mitochondria population in oocytes and make some special emphasis on the fact that the mitochondria in the oocytes are quite different than the sematic ones. And then I'm going to conclude about the potential of adding and replacing mitochondria, and how we can assess in the embryo the impact of such change or replacement.

So it’s started by oogenesis. It’s a process that lasts almost a year, starting with the very small oocytes to an elevated one, and this growth period is mainly taking place from the primordial or primary follicular stages, in that it takes months before this follicle is recruited for ovulation. What’s important to realize here is that it’s during that period of growth, of eight to ten months, the number of mitochondria will go up in the oocytes and will accumulate.

Once the oocyte is fully grown the stalk is already present. To illustrate that I have this cartoon where you can see, not really to scale but very similar, the size of the small resting oocytes. By the way, they stay in the ovary sometimes for 35 years, just stay like this at this size, and they have been built to that size before birth, so there’s just this kind of waiting period. When they start to grow the oocytes will go from 20 micron to about 120 micron in humans. So it’s six times, and that will take the six month or eight period that I mentioned. Once they’re fully grown, and they have this 100 micron, in fact they’re the size of the smallest dot you can see on a sheet of paper, that’s about the size of a human oocyte.

So you can see here these oocytes in the follicles. And the follicle is still small, maybe three millimeter, and it’s from that stage for example that the

patient will recruit for ovulation or will start to receive drugs that will stimulate the growth of several follicles to bring them to ovulation. So this follicle will go from three millimeter to 20 millimeter in a few days, about eight to ten days, and then end up in ovulation. And during that period the oocyte does not grow anymore. The mitochondrial components are mainly already acquired and don't change that much.

One thing I have to mention, I work mainly with cows and human oocytes and granuloma sells, and they're quite different than the mouse, because in mice when the oocytes are fully grown the oocytes are completely competent to go through fertilization and embryonic development. But in those two larger species only a subset of fully grown oocytes are competent, and that's still quite an important question, why not all oocytes are good. And that would resolve a lot of infertility problems if we really had the full answer to those questions.

One of the hypotheses is because the oocytes contain all the material to generate an embryo, which is a mass of totipotent cells that begin to grow at the wrong place, there's kind of the hypothesis that if the oocytes will self-activate like semic(?) models, all female mice die before their 12 month of age, stressing the fact that oocytes are dangerous.

So the ovary will not make them competent until there's a way to get rid of them, which is ovulation. So until there are LH receptors in these follicles the oocytes are not totally capable of becoming embryos. So I see it as a failsafe mechanism for the ovary to prevent something wrong happening before. So this LH receptor is a path to ovulation, it is also a very big signal, because then you have your follicle, which is epithelial, that has to change to a mesenchymal tissue to produce progesterone for the pregnancies. And this is also security for pre-pubertal animals, so they don't have those oocytes transforming into embryo or tumor within the ovary, and threatening the mother's life.

Quality factor. So by then you probably already know that yes, the size of the egg is important. It has to be full sized for the oocytes to have the capacity to even go through normal mitotic maturation, which is 80 micron in the mouse and 120 micron in human. The size of the follicles, it's really something that comes with larger mammals, and there's a huge effect of the level of differentiation of the follicle, and I'll come back into that in a few minutes, and there is the impact of age, which is not really present in animals the way it is in humans.

There is something special about aging ovaries

that we don’t see in other animals, and that creates a big problem which will come about as aneuploidy. Genetic factor, we’ve heard about that for the mitochondrial side, but there’s also a number of genetic factors in human that do affect oocytes fertility. Hormonal treatment, that’s what most people use when they go to IVF, and that is also an impact on quality of oocytes, mainly aneuploidy, again, as a phenotype.

Oocyte numbers. Just to explain to you the problem we have with super-ovulations, all species studies react the same with ovarian stimulation, more aneuploidy. This has been observed in all species where we’ve given them drugs to super-ovulate, the oocyte is often aneuploidic, is wrong. Largely it’s a mechanism to protect the uterus of the mother from too many fetuses.

It’s like the ovary knows how many spots there is in the uterus, and when there are too many follicles growing there is an alarm bell ringing somewhere that induces something in the oocytes that are going to create a faulty oocyte, and then the uterus can identify the aneuploidy embryos pretty well, except the down syndrome where it’s more difficult for the uterus to check them out, but most of the embryos are removed by the uterus, and the uterus destroys them actively.

The question that is really important for this

audience is if aging is something that we want to treat with different treatment, what is causing the aneuploidy that's going up with age. And as you may know there is an exponential rate past age 32. It's not linear as before. So there's something happening there that's not just the effect of time. Much more pronounced in human than animals. Like menopause, could that be an adaptive mechanism?

So the oocytes are made wrong, so the women don't bear children after 30 years of age for evolutionary family reasons which will help the survival of the maybe already born? And we have data from bovine oocytes, because we can capture oocytes and extract RNA from them. It's much easier in bovine than it is in humans, and you can imagine why. And we find a number of spindle regulation problems with oocytes coming from compromised follicles. So we know that the ovary can change the oocyte quality when something is going wrong.

This is a graph of the increase of aneuploidy with age. You'll see a very small rise between 25 and 35, and suddenly start to go up. And at the same time implantation rate is going down because the uterus can detect the aneuploid embryo and remove them. But more striking is the exponential rise, this is Down syndrome, so it really goes up the roof by the time people are 35 years of age. So there's something active in the ovary creating

this phenotype, and people would really want to know what it is to address how to treat that.

So coming back to oocyte competence, I would like to stress that it’s not often present. Less than 10 percent of the oocytes that are recovered from patients end up in babies, or embryos. Per oocyte basis this is a very low success rate for a species that is overcrowding this planet. What I want to stress as well is the competence goes up and down quickly. Within a two day period the quality of oocytes can go up and down, I’ll show some evidence for that in bovine and humans. The competence is compromised by stimulation, creating more aneuploidy in all the species that have been studied, and also because the follicles are often ovulated a bit earlier than you would have normally at the smaller size, because of the effect of the drug, the oocytes have less differentiation status in super-ovulated context than regular ovulation.

So we’ve been working the last 20 years on the follicular determinants of oocyte quality, and what we’ve begun to understand is that as the follicles grow and differentiate there are a number of signaling that are transferred from the environment of the follicles towards the cumulus and the oocytes, translating that into capacity of these oocytes not necessarily to fertilize, fertilize is like an autopilot and it normally occurs much earlier, but

what makes the difference is the signaling here that controls the embryonic development afterwards. So it’s the background of the oocytes, the molecular background containing the oocytes that make the difference between the good and the bad oocytes. It will not show in terms of cleavage, it will show here in terms of embryo formation.

So in a nutshell the data over all those years was simply to show that there is a very low competence of the oocytes to become an embryo during the FSH stimulation period. When the follicles become dominant, where the LH receptor appears, there is a big jump in capacity, and at the end when the LH pulsing creating the ovulation cascade, then the oocyte reaches maximum capacity to become an embryo. And those two last things, or step up, happens within three or four days of ovulation. So there is a very rapid change in oocyte quality within a few days before ovulation.

I therefore believe that the oocyte quality is influenced by the follicular differentiations status. And the oocyte’s potential is determined before fertilization. These are three studies we’ve done with our human individual aspiration of follicles with the Ottawa Fertility Clinic a few years ago, and we have found a number of markers.

We can predict based on the follicular

transcriptome how good are the oocytes and how are the chances of the oocytes to become an embryo, showing that the ovary controls quality. We even have some markers that can predict failure, so the ovary can induce failure. So that's interesting if you're trying to understand how to treat infertility. And we have the same phenotype of course, as has been observed in cows.

This is only one graph to capture all this. It's individual follicle aspiration, this is follicular size. I just wanted you to focus on this one. This is the follicular size as measured by volume, and you can see the bell curve of embryo that are going forward and being transferred, 7-8 cells embryo is going up as the follicular volume is going up, and is going down when the follicle is getting too big. So the ovary has a way to turn this off. We don't understand exactly how it works, we have markers now we're setting. But the point is the ovary controls follicles(?).

Now, I have to address the mitochondrial aspect, even if I'm no mitochondrial expert, I must declare. And it has been already shown that the mitochondria are kind of passed along in the primordial germ cells. So a few days in the mouse after fertilization, or a few weeks in humans. Becomes an oogonia, and then primordial follicle. They don't grow, they stay like that for 40 years. The growth

period starts with primary oocytes, and that's where the bottleneck here is, and then as the cells grow it increases the number of mitochondria within this 8 month period.

So during that amplification from primary to mature oocytes there will be a huge amount of amplification, but then as it's mentioned, once fertilization occurs through segregation, and only a few cells from the inner cell mass will become primordial germ cells, and will transmit these mitochondria to the oogonia that will create the next generation.

You've seen this slide before, it's how the component of the oocytes that are heteroplasmic will be transferred, not necessarily always in the same proportion, because of this bottleneck period where whatever selection procedure occurs creating different proportion in oocytes. What we don't know and I think hasn't been addressed is how much there is variation between different oocytes in the same ovaries in the same patient or in the same animal, I don't think this has been addressed on an individual oocyte basis, but that would be interesting to know.

I would like to stress the differences in oocytes' mitochondria. They're very special, they don't look like regular mitochondria, and they're potentially in a sleeping state because they have a different morphology, they have intense selection pressure on them. They have

potentially low membrane potential, but there is still controversy about that. And they have a number of ATP enzymes that are reduced during maturation. And we see a number of energy pathways that are activated that don't require beta oxidation.

So these are oocytes mitochondria with electron microscopy, and they have a very different shape, they have those hook form, they have vesicles, they have much less cristae, this is a control one, and therefore it's been hypothesized that they are less functional, they're not as good machines for burning oxygen. In fact there is not much oxygen around oocytes, so maybe that's the reason they have to switch to other sorts of energy. And also in our polysomic analysis of oocytes during maturation we've seen that there's a number of protein in the different complexes of respiration that are down-regulated. So it seems that the mitochondria are kind of shut down for a few days until the genomic activation resumes. So that's interesting to know, although we don't really explain that.

Here is a picture from one of the mitochondria experts, Jonathan Van Blerkom. And I would like to stress on here that distribution and the special shape of mitochondria, we can see them again, that are really different, and here I want to stress this is a metaphase plate, and you can see by the tints of the coloration where

the mitochondria are, and this is a pro-nuclear status. So they're really around the nucleus and the metaphase, which is interesting.

Here are blastomeres, and what this paper shows is that there is unequal distribution of mitochondria, often in the blastomeres. So when you do biopsy to analyze the competence of one you might miss what's happening in the others. So that's a warning that blastomere might not be equal in terms of mitochondria. We can also use stain to evaluate the potential, but again there is variation in terms of are the mitochondria active on the periphery of the eggs because that's where there is oxygen, there are a number of questions that have not been resolved so far for measuring mitochondrial activity.

This cartoon really illustrates for this panel, for this committee, the segregation of oocytes during maturation to see how much they are around the spindle during maturation, afterward how they co-localize also with the pro-nuclear. Because also these spindle and pro-nucleus, because a microtubule needs a lot of energy, explaining maybe why there are so many mitochondria. After that they seem to segregate to the periphery of the cells, facing the environment.

You've seen this slide before. Manipulating the mitochondria, there are two different approaches. There is

replacing them all, but all with this picture I show you will become a matter of measuring if there's anything left. Spindle or pronuclear transfer, or injecting new ones or cytoplasmic transfer, either from oocytes or, and I think my big worry is here, from somatic cells.

I guess another speaker will explain more into that on what happens when we do a spindle transfer. In fact, the resting mitochondria are maintained there and the spindle is transferred from one oocyte to the other, pronuclear transfer is the same thing but with later stages where the sperm has entered and form a pre-nucleus. It's two basic ways to create that.

What is a bit more controversial or difficult to apprehend is when you do cytoplasmic transfer by mixing the population of the endogenous oocytes with the exogenous cytoplasm, creating heteroplasmy, to see if this heteroplasmy will be sufficient to treat disease, sufficient to address aging problems of mitochondria, if any. So that's a more problematic approach for me.

We've been playing with those type of thing in cows using two different breeds, bos taurus and indicus. The transfers survived, but what I want to show here is that the bos indicus transfer in the bos taurus oocytes, as developmental time goes to birth, those mitochondria are selected out in growing fetuses. So there is some selection

mechanism occurring, maybe which is because there is too much difference in those two species, creating less functional synergy between nucleus and mitochondria, creating a natural diminution of the exogenous mitochondria.

We also played with transferring somatic mitochondria in oocytes, because in animals there is much less issues so we can start playing with that. And what I want to show with this slide is simply that. When we do use mitochondrial blastomere versus sematic cells, what we see is that the mitochondria from sematic cells are gradually removed compared to the one from blastomeres. So there’s enough difference between these sematic and oocytes mitochondria, so there is a different fate of those mitochondria when they’re mixed together. So the oocytes mitochondria or the embryo mitochondria will survive better than the somatic one, being added.

How we play with functionality to see if those mitochondria are either functioning well or not and what’s the consequences of inhibiting their respiration or stimulating their respiration, what’s the effect of ovarian stimulation, and we know that there are patients with a very specific problem, and the next slide will illustrate that, where we have very defective mitochondria with dense granules in them that creates un-functional mitochondria.

So these cases are possible and they do arise, and those cases probably have a nonfunctional embryo, in those cases asking for a complete replacement because those mitochondria cannot go any further. But if we push beta-oxidation by L-carnitine supplementation, we show that can increase the functionality of mitochondria, and in this mouse model can increase developmental competence. So sometimes having more energy seems to be making a big difference for embryo. So mitochondria might be therefore a sign that if there's non-functionality there could be consequences in terms of competence to develop to an embryo.

Another warning here is mitochondria and controlled ovarian stimulation. This investigation shows that when we stimulate with drugs and use in vitro maturation this treatments might inhibit the final mitochondrial replication in the last few days, although for me they're already formed, but this is potentially a last window for application. Damage related to the mitochondria may explain the low efficiency of IVF, because we use a lot of ovarian stimulation in those oocytes. So there is maybe an association between ovarian stimulation and less functional mitochondria.

Part of the equation, oocytes of course with nonfunctional mitochondria cannot be a viable embryo,

that’s clear. But conversely a nonviable embryo may have functional mitochondria. So it doesn’t mean that because the embryo is not doing well that there it a mitochondria problem. Mitochondria is just a small part of the infertility equation. And what is the ratio of embryos that failed? Ninety percent of the oocytes that are aspirated in patients do not become babies. So we still have a long way to go to understand the oocytes’ quality issues here. So what is the ratio of embryo that failed due to functional mitochondria? I don’t know, and I don’t think we have the experimental design even in animals to address today this question.

I’ll finish with how can we select embryo if we’ve made a modification to them. There are different techniques now that are emerging, like morphokinetics, where we can assess the speed of cleavage of embryo, and that has pretty good selection in terms of their capacity to become babies, but it’s not 100 percent, and it’s mainly insufficient if you want to address the quality of an embryo unless there’s an obvious phenotype, and if the mitochondria does not affect the speed of cell division for example you might miss completely the potential defect that the change might have, because the embryo will divide quite normally, it’s kind of automatic pilot.

Another system that is used mainly for aneuploidy

screening is called CGH, and it mainly is measuring the amount of chromosome material for all 23 chromosomes, and when there is over-representation of one of those chromosomes you can see you have aneuploidy. So this could be useful to screen aneuploidy if that would be the consequences of mitochondrial manipulation. This could also detect metabolic mismatch. This is important. With the same cells now we can do transcriptome analysis, and for example if we take a bovine embryo, they're very sensitive to glucose, and we give them five millimole of glucose for two days before they reach the morula stage, they're going to get sick.

Their mitochondria is going to be totally perturbed, and the way they're going to respond to glucose in the blastocyst stage is going to be different, and we can have a transcriptomics and epigenetic signature, so when we mess up mitochondrial function early on we see the effect of these on the blastocysts. So if people are looking for markers of the perturbances of the mitochondrial in nuclear interaction early in embryo, transcriptomics and epigenetic analysis could potentially answer part of the question. And of course the biopsy could be used as well for mtDNA analysis for a mutation.

To conclude, animal models, total replacement. Mouse has been shown to work, monkey also has been shown to

work. Infertility treatment, adding mitochondria, I may be in conflict of interest for this one, bovine is probably for me the best model for generating oocyte-like stem cells and testing them on experimental oocytes-embryos-fetus-term with all the tools we have to address if there's any potential problem with using exogenous, somatic, or oocytes derived mitochondria injected into oocytes.

So to conclude on competence, age, and ovarian stimulation impact, mainly through aneuploidy, competence is labile. Incompetence may be age-induced, innate or patient specific, acquired during oogenies, or acquired during ovarian stimulation. Mitochondria functionality is essential. Stimulation or inhibition of mitochondrial activities impact embryo quality. If transfer is used chimerism is possible but not lethal. Distribution is difficult to predict as selection occurs, and the first two talks have put some emphasis on that. And that's it. Questions?

DR. SNYDER: We are open to questions.

DR. MORAES: When you transfer mitochondria you are actually transferring ooplasm, right? So how do you know it's the mitochondria or not something else in the soup?

DR. SIRARD: Of course we are transferring all part of organelles, reticulum, endoplasmic, but those

things, not only because they don't have their own DNA, will not last for a long time. So the worry is it is the mitochondrial genome that has lasting effect. Other than that we've seen in embryos, many fragmentation, if the embryo doesn't like something it's going to make a bubble and push it out, and even the more aneuploidic cells will go into trophoblast, and the embryo is kind of selecting the best ones for its embryo proper. So there's a lot of dynamic activity in embryo to get rid of what it doesn't want.

DR. MORAES: But it could be something else, right? Some molecule that's present in better oocytes could have some trophic factor or something like that.

DR. SIRARD: If you use a cross-species transfer that would be a bit more worrisome. But within a species I don't really see any major problem of fusing other components of the oocytes within other oocytes.

DR. MORAES: It isn't a problem, I'm just wondering if it's the mitochondria or something else.

DR. KEEFE: Assisted reproduction has been associated with considerable epigenetic changes. It appears to be related not just to the culture, but the group from Texas showed the nuclear transfer itself is associated with marked alterations, the large calf syndrome, epigenetics. Your own work and other shave shown that TET enzymes are

very important for regulating methylation status in the early embryo. Does the redox state, and therefore changes in mitochondria oxygen tension and so on, do they affect the TET dioxygenases? Could this be a potential mechanism, and maybe also you could just more generally discuss the epigenetic concerns in engineering oocytes.

DR. SIRARD: We have done a lot of transcriptomics studies to show either lipids, free radicals, and glucose are impacting on embryos, and all three components do have a huge impact on transcriptomes. So the embryos react rapidly to its environment. What we don't know but now we're getting the tools to do that is the methylation status. So for example if a particular factor four is a factor that is affected by glucose in embryo, surprisingly that's the same factor that is affected in a diplocyte of people who have developed type two diabetes.

For example they use twins, homozygous twins, one has diabetes, the other does not. They look for differences in methylation in adipose tissue, and one of those genes is the same that is reacting to early embryo. So for us it's a potential way to say if we change metabolism early on we have a legacy, we have a change that's going to be carried to birth and may impact on the future of this embryo. That is the new field of epigenetic and embryo, and mitochondria can play a role in that, is the amount of energy received

by the embryo is impacting the rest of the life of the future children. The answer now is yes.

GOLDMAN: Following up on that question. So in the setting of adding mitochondria for the purposes of increasing viability of a given egg, is there good data out there in terms of the effects on the redox state of that recipient egg? And then expanding the question that was just raised in terms of effects on TET and therefore epigenetic state, just in terms of redox effects on thresholds for tyrosine kinase, and kinase effects downstream signaling cascades in cells. There is a large literature of course in terms of effects of redox changes on those cascades.

And so I wonder what kind of end points are available that allow one to assess what redox state is in these cells and to what extent, thinking in terms of the morphokinetic output or endpoint used in one of your slides. Has that been correlated, have these different outcome measures been correlated in terms of effects of redox state on tyrosine kinase activity, and therefore map kinase dependent cascades that are involved in proliferation of differentiation. Are there any data available that correlate that to the kind of morphokinetic endpoints that you’re using?

DR. SIRARD: The way we can play with redox state

is mainly using beta-oxidation stimulation like L-carnitine in culture media. That's so far in the literature what's been mostly used to address if indeed there is more free radicals forming, and the way to address that often is difficult to measure in an embryo. What we do is there is a detrimental effect that is covered by anti-oxidant. So what we see as when we stimulate beta oxidation the need for anti-oxidant is higher, so therefore if you don't give anti-oxidant and you raise beta oxidation it's detrimental to the embryo. But except for that if people use exogenous mitochondria to crank up the oxidation and redux, I don't think I've seen any paper in that direction. It's simply by chemical manipulation again, addressing the question you asked.

DR. SNYDER: I guess I have one quick. I want to clarify one of the points that you made in one of your slides, because it might get to one of the practical aspects we need to get to in the afternoon. In your slide you suggested that trophoblast biopsies could be a practical way of looking at mitochondrial DNA analysis. Is that correct?

DR. SIRARD: When you do biopsy you can get the RNA to look for any metabolic perturbances from these new mitochondria. You can look at the epigenetic response to the metabolism change within the trophoblast nuclei, and

you can look for mitochondrial DNA mutation because you have the sample. But it doesn't mean the picture that you're going to have of the trophoblast is totally reflective of the mitochondria population that's going to be in the inner cell mass, so that's the limitation.

DR. GEARHART: One last question, there was a paper published as I recall from someone at our institution that dealt with the redox state at the pronuclear stage in mouse embryos programs body weight in adults. Are you familiar with that? Is out there

DR. SIRARD: There are experts, Monachelli(?), for example, has studied the effect of manipulating the diet to create disease mitochondria that have this different redox state at the pronuclear stage. And one of the phenotypes or mice that are going to be more prone to be obese afterwards. But the mechanism behind that is simply unknown. We think it's epigenetic, but the mitochondria is slightly modified by methylation, so it might be nuclear mitochondria interaction that result in the imprinting or programming of future disease in children.

DR. GEARHART: But the issue is that the redox state at that point, which is one of the stages at which some of these manipulations could be done, could have a dramatic impact downstream.

DR. SIRARD: In animal models, especially looking

for the transmission of obesity across generations, now we know it's not really genetic, it's epigenetic, and there are a lot of models showing that the embryo is sensing the environment two times in its life, first in gamete formation and the second time post-fertilization, it's very sensitive.

DR. SNYDER: Any other question or comment? We're ready to move on to the next presentation from Dr. Latham on epigenetics, inheritance, and assisted reproduction technology.

Agenda Item: Epigenetics, Inheritance, and Assisted Reproduction Technology

DR. LATHAM: So the question session nicely sets up the last part of my talk. So if I don't come back to things enough definitely let's come back and visit those questions in more detail. The work of my lab really focuses on early development, basically the first few days of life. I guess looking for a place to begin my research I decided to take the advice often given, the best place to begin is at the beginning.

So I begin at the beginning, I'm still at the beginning because there's so much to understand what's going on. It is truly a unique part of life. You bring the two gametes together, and that will initiate the cleavage to the formation of a blastocyst. And the embryo during

this period is really different. These aren't just big somatic cells. The early embryo and on through early cleavage divisions has different metabolic properties, has different mechanisms for ultimate regulation, it has different mechanisms for cytoplasmic pH. Regulation, it has different carbon substrate requirements.

At the very start of life there is no ability to regulate gene transcription. The ability to regulate gene transcription is acquired during the second cell cycle. There are genomic retrotransposons, genomic parasites that become activated and have to be rapidly shut down. The program has to be set in motion to initiate this wonderfully complex process of forming an embryo that's able to implant into the uterus and have the appropriate dialogue with the mother.

And during all of this we have vast reprogramming of gene expression. The gene expression profile, the first two cell cycles is profoundly different from any somatic cell you would care to look at. So the vast reprogramming of gene transcription, the vast reprogramming of protein expression, there is a global change in DNA methylation, superimposed upon that of course you must maintain genomic imprints to keep that information going on to control your development correctly.

So it's really a profoundly important period of

life. And a big part of what I'm interested in is understanding the mechanisms of this reprogramming, and understanding the consequences of these changes for later in life. So basically there's a lot going on here to connect environment to adult phenotype. This is a really important new area of biology, this developmental origins of disease has come up in the last five or six years.

So as the embryo grows cells differentiate and you go on to have adults with various diseases, and then they have to go on to reproduce. A lot of these phenotypic characteristics are determined by things that happen back here in this time period. And a really good example, just keep this in mind, a really good example of the importance of the link between this stage and adult phenotype is that if you restrict the protein intake of a mother just during the pre-implantation period, just reduce the protein intake by half, balance it with calories, and then provide a normal diet after those first four days in the mouse, the progeny end up being hypertensive. So just a transient, very modest change in maternal diet is enough to profoundly affect the postnatal characteristics of the progeny, and that same thing has been observed in other species as well.

So we have genetic factors that control development, we have epigenetic processes that control development, we've got genetic factors that control the

epigenetic processes, and then superimposed on all this is the environment, and the environment can be reproductive toxins, it can be maternal nutrition, it can be stress, and of course for the purposes of today's conversation it can be clinical procedures, embryo manipulations and oocyte manipulations. So I just want to start off by really impressing on you the unique nature of these early days of life, and Marc-Andre mentioned to you the unusual mitochondrial characteristics as well for today's discussion.

So I just want to quickly review some things that came out of some of our early studies. I'm not going to have time to go into these in detail. Basically, some early studies using pronuclear transfer revealed that the epigenetic modifications of the parental genomes continue to be modified after fertilization.

There's sort of a perception that imprinting happens in the gamete. Well, that's part of the story, but allele specific changes can also arise post-fertilization, it can arise all the way up until the time when those two gamete genomes are united into a singular genome. So we've published studies showing that different strains of oocytes will modify paternal pronuclei differently, and that affects the development of uniparental embryos.

Another study early on suggested that intra-

strain nucleocytoplasmic hybrids can display growth defects. These would be embryos produced by swapping pro-nuclei between eggs of different mouse strains. And I just want to point out then that the kinds of clinical procedures we're talking about, cytoplasm transfer, spindle transfer, GV transfer, and so on, do in fact result in nucleocytoplasmic hybrids. So the question arises what might be the epigenetic risk of these procedures.

We did a series of studies a while back where we did maternal pronuclear transfer, maternal oocyte cytoplasm transfer, and spindle transfer, using two different strains of mice. So we did either inter-strain transfers or intra-strain transfers as controls. And for most of those procedures we saw no real effect on growth rates. The growth rate was something that had been pointed to in a previous study as being potentially affected in nucleocytoplasmic hybrids.

So we saw no effect with the cytoplasm transfer on growth. That doesn't mean there weren't other effects, it just means that we didn't see effects on growth. We also didn't see effects on growth with spindle transfer or maternal pronuclear transfer. I will say though that the viability of those embryos is reduced, so there is some negative effect of it. We are seeing that the viability reduction is sufficient perhaps to cull out any problem

embryos, so we didn't see any effects on growth later. Where we did see an effect was with germinal vesicle transfer.

So GV transfer, so you've heard about cytoplasm transfer and spindle transfer and pronuclear transfer, GV transfer is taking it back one stage earlier. This is now taking oocytes that have not resume meiosis and exchanging the germinal vesicle at this early stage. So this would be another way of accomplishing a complete change of cytoplasm, and theoretically a complete or nearly complete change in mitochondrial composition. And this was suggested a while back as a potential therapy to be used in the clinic.

And we did see a significant decrease in growth rates in the female progeny following inter-train GV transfer. We did not see this with intra-strain GV transfer, and we did not see it with the non-GVT IVM controls. And we didn't see it in males, so this affect was specific to the female progeny from the inter-strain GV transfer.

It just really raises the question then how can something as seemingly benign as doing a GV transfer at this early stage lead to an effect on something profound as growth? Growth has to be one of the most basic fundamental phenotypic characteristics of any organism. It's affected

by a vast array of genes, a vast array of processes. It is in some ways a very good sort of biological readout of things gone awry during development.

But nevertheless there is the perception that eggs are eggs. Well, apparently not all eggs are equal. So we have these interesting genetic differences that we can exploit with the mouse. So basically cytoplasm transfer was not associated with pronounced group deficiency. We also measured urinary protein and other imprinted gene methylation profiles and saw no change there. We also saw no effect with cytoplasmic transfer or pronuclear transfer.

But with the germinal vesicle transfer we had this growth deficiency in the females, and again though we did not see an effect on the measured urinary protein or any of these other imprinted genes that we looked at. I'll also point out that the growth efficiency was seen basically at one week after birth, so it doesn't appear to be related to any growth hormone deficiency. This is something arising really from processes happening all through fetal development.

So I just want to sort of go back and review some of the stuff that's been published now on mitochondrial transfers to sort of set the stage for what I want to get to in a few minutes. So a lot of really nice studies have been done over the years using cloning, somatic cell

nuclear transfers, and other kinds of experiments to look at what happens to mitochondria in manipulated embryos.

Basically, in some studies with cloning the daughter cell mitochondria become eliminated. In some studies they can persist. In some studies they can actually increase in abundance. And I think one of the cloning studies I think was rabbit nuclei into mouse eggs, the rabbit mitochondria actually took over, they pretty much came to be 100 percent of what was there later on in development.

So there’s a lot of variation in our cloning experience, there are results all over the map. I don’t think we really have a good idea what controls the outcome of that, is one of the more interesting aspects to the early embryo to think about. There are reports that heterotypic mitochondrial combinations in cloning can be a disadvantage. There are reports that different haplotypes of oocyte mitochondria can affect development of early bovine embryo as well.

From the nonhuman primate my lab published a report showing that oocytes of different quality have different expression profiles of mRNA encoding mitochondrial proteins. Other reports reveal mitochondrial impairments commonly seen in low quality oocytes. Other experiments, as Marc-Andre indicated, intra-species

mitochondrial transfer, again sometimes they can be eliminated, but in some cases the heteroplasmy can be disadvantageous. And there was another study reporting that serum-starved mitochondria can inhibit pathogenetic embryo development.

Other studies reported that granulosis cell mitochondria can be injected into the early embryo and they can enhance development and blastocyst quality of bovine embryos when you're starting with poorer quality oocytes. Mitochondria can be injected to rescue ethidium bromide treated embryos. Mitochondria can be used to improve oocytes from aged mice. And then a couple of anecdotal reports in the human literature of children being born following mitochondrial injection. And then more recently this report that spindle transfer can be successful as well.

So on the surface it looks like mitochondrial therapy can be good. So why is Keith up here trying to tell you about potential concerns? And I think one of the really interesting studies that came out in the recent literature was this study from Richard Shultz and Jean-Pierre Ozil collaborating. And basically what they found was just that by varying the ratio of pyruvate and lactate within the embryo culture media they were able to affect the growth characteristics of the mice after birth.

So they could starve the embryo for a certain period of time, take away both pyruvate and lactate, or they can increase the pyruvate concentration or increase the lactate concentration, and they can really make the mice get big or make the mice get small. They can do the same thing with phosphorylation alkalinization.

And what they showed is these treatments change the redox state of the early embryo. If you read the paper carefully you can sort of come up with some basic rules that if you decrease mitochondrial activity you tend to get reduced growth, if you increase mitochondrial activity you tend to get enhanced growth. There are some complicating factors. There can be a litter effect with some treatments, but not with others. There can be a sex effect with some treatments, but not with others. But it really just drives home the point.

And we’re not talking about long-term treatments. They’re able to observe changes in the redox state within as few as ten minutes of change in the culture medium. And for the embryo transfer studies that they did I believe they only applied these different media for about ten hours. So basically a relatively short-term transient exposure of the embryos to these slightly different medium formulations was able to have profound effects on the growth of the mice after birth.

So you can think about how this can happen. There are many possible explanations. The redox state is tied to the activities of some very interesting transcription regulators and chromatin modifiers, such as these sirtuins which use NAD as cofactors. The redox state is also going to be controlling availability of ATP for ATP dependent processes such as chromatin remodeling. Outside of that there's a sort of general overall miasma of this as yet undefined observation that a wide range of relatively mild stress on these early embryos can have these kinds of effects on fetal growth and post natal characteristics. So this appears to be one part of how those effects are emerging

So just to start summarizing, these oocytes display genetic differences in composition. The maternal genome and ooplasm must be compatible. The paternal genome epigenetic modifications are variable, and they can be changed after fertilization. Early stress to the embryo leads to changes in growth and physiology. If you change the redox state you can have long-term effects on growth characteristics and post-natal phenotype. The localization of the mitochondria within the early embryo correlates with embryo quality and developmental potential.

Some of the questions that come up from these observations are: How might somatic cell mitochondrial

injection effect redox state? You can certainly envision, Marc-Andre was mentioning the difference between oocyte mitochondria and early embryo mitochondria compared to somatic cell mitochondria. So if you inject mitochondria from somatic cells you can be profoundly affecting the redox state of the cell, which raises concerns given this study of Ozil and others about what you might be doing to the long-term effect on the embryo.

Other questions that come up: Are there going to be required matched between mitochondrial and nuclear haplotypes? What is the impact of the patient's mitochondrial haplotype? Again, going back to some of the bovine studies suggesting that mitochondrial haplotype in the egg affects developmental potential and embryo quality.

How pure is the mitochondrial preparation being used? So when you go in to isolate mitochondria from somatic cells, what else are you getting out with those mitochondria? What might those other factors be doing when you inject those into the eggs? And all this sort of comes to bring you around to question what are the potential epigenetic effects of these procedures, and potential incompatibilities that you might be setting up. When you look at the published data I encourage you to ask how predictive are the data from studies whose N points are to look at parthenogenetic blastocyst formation, as an

example. I would argue that those are not terribly informative N points.

How informative is blastocyst formation frequency, blastocyst characteristics, these kinds of things? I would argue that those are not necessarily the most informative N points that need to be considered for this conversation. We'd also get into questions about the overall efficiency of these procedures, and potential cost concerns.

Animal models can be really valuable in this context. With the animal models you can track long-term phenotypic consequences, particularly if you use animal models with shorter life spans. If you start moving into clinical trials I would say the end of the experiment is not cleavage, it's not blastocyst, it's not pregnancy rates, it's not term deliver, the end points of clinical human trials are going to be coming up decades later, because it's going to take us that long to find out potentially what are going to be the effects on the health of the progeny produced from these procedures.

This is why it's valuable to use animal models. We use animal models with shorter lifespans, we use animal models where we can consider perhaps the most extreme manipulations or the most extreme combinations possible, and try to extrapolate back to what might or might not

happen with the human population. We can deliberately vary mitochondrial nuclear genetic combinations.

You can't do that really very well in the humans. We can go in and analyze oocytes of embryos before and after these manipulations. You're not going to be doing that, I don't believe, in your typical clinical practice. You can use the highest quality embryos for these studies. You're not going to be limited to using oocytes that have failed to fertilize, or oocytes that are judged morphologically failure to the ones that are used for more conventional methods. With these animal models you can use the very best quality material, you can use a consistent homogeneous quality material so you can get good replication from study to study and follow what's happening long-term.

You can collect fetal and post-natal tissues to assess the epigenetic effects. You can use genetic tools. Mice have some, other species have other that you can use to eliminate certain experimental barriers. And you're probably going to be wanting to look at multiple models to address what might be happening with these procedures. The mouse is a great model in some respects, it's a not so great model in other respects. So combining the mouse with the bovine or a nonhuman primate will be a really good thing to consider doing. Any questions?

DR. PERA: On your last slide I guess I would really question, and you're the one to really answer this Keith, I have my doubts on the mouse as a great model, and I think that nonhuman primate might be superior. Could you just spend a little bit more time talking about that?

DR. LATHAM: So the mouse, the advantages of mice is you have genetic strains that you can use, so that allows you to do the same experiment over and over again. If you use an outbred population you can't ever really repeat that experiment, you've lost that unique genotype. So with the inbred strains you can repeat that experiment as many times as you want. Short lifecycle, very well established methods of culture, we know what works well, what doesn't work so well, we know how we can sort of muck things up in a very deliberate controlled fashion and how we can avoid mucking things up in a very deliberate and controlled fashion. So there's all this beautiful background available with the mouse.

But the mouse is a litter bearing species. It doesn't have the same ovarian physiology, it doesn't have the same reproductive physiology as humans. Cow is a much better model than the mouse in this respect, and nonhuman primate is an even better model in this aspect. Rhesus monkeys require relaxin stimulation for highest quality blastocyst development. The mouse embryo doesn't have a

relaxin receptor at that stage. I can stand here and go on for hours about the differences between the models.

So the mouse has the advantage of genetic tools, genetic power, rapid turnaround, you can look at transgenerational effects in a relatively short timeframe. I think it's great for looking at and defining what things can happen, and then take those data from the mouse and go and expand upon those in bovine or nonhuman primate to find out what really is happening in those models.

DR. PERA: I agree, but I do think a lot of things just don't happen in the mouse, and one really great example is aneuploidy. We've tried to develop mice that are aneuploid like humans and it just doesn't happen. So I think that it would be reasonable to be very cautious about both bad and good outcomes with mouse and how they might be related. There doesn't seem to be too much, and I'm not advocating particularly nonhuman primate research, but there don't seem to be great differences between the nonhuman primates and humans as with mouse, obviously.

DR. LATHAM: I think defining some of the basic perimeters such as asking how the redox state of the zygote affects long-term development, I think those things can and should be done first in a mouse, because they can be done quickly, we can get rapid data on lifelong effects and transgenerational effects and then take those back into a

larger species.

DR. KEEFE: My favorite quote about that is all models are wrong but some are helpful. Clearly you've done a lot with the mouse. My question is about the results you showed in nuclear transfer and the effect on weight. One of the interesting things that the Texas folks showed, and others, when they looked at nuclear transfer in the large calves' syndrome, was not only an effect on the average rate, but the variation increased. That's really interesting.

It significantly increased, up to a fourfold increase in variation from small to largest. I'm wondering A, did you see that in the moues, and B, what's that telling us. IT's important as you move into a human, because it's going to be trickier, you're going to use an F-test rather than looking for differences in the meme, and it could be hiding major important differences.

I'd rather have a basketball team with everybody averaging six feet but some 6'8 and some 5'8, it's going to have a difference performance than everybody averaging. So it could be flipped around, there could be some detrimental effects that get hidden in the mean not changing. Did you see any change in the variation? And what do you think that means biologically when the large calf syndrome is associated with an increase in variation in weight, not

just average?

DR. LATHAM: So we have not seen these kinds of growth effects in our mouse cloning studies. The large calf syndrome and the large offspring syndrome is something that has been there in the literature pretty much since people started making cloned cattle. My understanding is a lot of this is tied up with particular medium components, as well as other procedures involved in generating the oocytes, embryo culture, conditions, these kinds of things.

It's a complex process leading to this. I remember having a talk with Marc Lestusian(?) a while back, and it seemed like they were able to eliminate a lot of these problems by refining some of the methods. This is always the problem with this field, there is a wide variation from laboratory to laboratory, protocols, operator dependent skill levels become part of it. There are a lot of variables that go into this large calf syndrome. I will say that what I've heard is that some of the biggest problems with the cloned calves are the respiratory distress that they go through. If you can get them past that they go on to be very normal individuals. You sort of have to get them over that bump early on.

DR. MORAES: If you take mitochondrial from somatic cells and inject it into oocytes, do they do better? What's the experience again?

DR. LATHAM: I haven’t done those experiments. I have to shamefully admit I haven’t reviewed the literature in that depth before coming here today, but those studies are out there, you can look at them.

DR. GEARHART: You certainly give us reason to be circumspect. Almost everything you touch early on with the pre-implantation embryo there may be oversight we have to take a closer look at. There certainly are parallel situations when we think about the rationale of stem cell therapies, of what kind of cells are producing and how long are they going to work, and are they going to do something untoward. But here we are dealing with essentially the production of a human life, a person, and the impact. In any of the mouse work are you aware of any kind of cognitive tests that have been done on any of the animals that have been generated in these ways? Memory or learning for example?

DR. LATHAM: I think Richard Schultz published some data a while back with his embryo culture experiments to suggest that there were cognitive impairments with embryos grown in Whitten’s medium as compared to KSOM.

DR. GEARHART: One thing that didn’t appear in your slides are for these different types of manipulations the issue of embryo loss as a direct result of physical whatever or interrupting centrosomes, is this high under

the state of art at this point, in the mouse at least?

DR. LATHAM: In the mouse pronuclear transfer is really pretty efficient, cytoplasm transfer is reasonably good, there's a little bit lower because there's two manipulations. Spindle transfer we had quite a bit of loss, survival of the actual fusion of the couplets. With the GV transfers I think about half of them survived the manipulation, and then we would have some loss going on through early cleavage, and then we have quite a bit of loss after embryo transfer to giving birth.

I think we wound up with about eight percent going on to birth with that procedure. So the efficiency with these kinds of methods is a concern. I've been told that the human embryo was much more forgiving of micromanipulation than the mouse embryo. So different numbers would probably apply in that case.

GOLDMAN: Following up on John's question with something maybe a bit more sci-fi. If you alter the redox state of a given egg, let's say in the setting of a pronuclear transfer, and then impart of course a new set of epigenetic marks as a result, one generation later can the male progeny of the resultant individual conceivably propagate that epigenetic mark? Is there any mouse data to that?

DR. LATHAM: So my lab specifically has not done

those experiments. I don't think anyone has done those experiments specifically in the context of redox state. There are other examples of transgenerational inheritance, some of the stuff involving toxin exposures in the testes for example, Mike Skinner's work. Another series of experiments that we did involve looking at embryo fragmentation, and we did various kinds of maternal pronuclear transfer experiments with that, and we were actually finding that the fragmentation phenotype at the two cell stage was being affected by the maternal grandfather's genotype, suggesting some kind of transgenerational epigenetic information coming through there on that effect.

The whole question of transgenerational inheritance I think is something that's just now starting to be looked at in more detail. So I think as we start seeing more documentation of these kinds of epigenetic effects then people are going to more and more start following that up with transgenerational study. I think we're just early on time towards some of these studies though.

DR. SNYDER: Any other comment or question? The next presentation will be from Dr. Mitalipov, and will be mitochondrial genome replacement in unfertilized oocytes for treatment of inherited mitochondrial DNA disease.

Agenda Item: Mitochondrial Genome Replacement in Unfertilized Oocytes for Treatment of Inherited MTDNA Disease

DR. MITALIPOV: As we heard the mutations of the mitochondrial genome does cause a variety of diseases in humans, and actually manually counted the number of mutations that have been reported to a cause specific condition in humans, and there have been more than 700. Doug Wallace actually tracks them in his website, mitomap.org. So of course some of them are inherited. That means it came through maternal linage, but the others are acquired, age related, so they're more in somatic lineage. So of course the focus today is on inherited mitochondrial gene mutations.

So as we heard the inheritance actually, the mutation is very complex in mitochondrial genome. For example, as you can see here the symptomatic mother, she could have children that actually have a mutation, but the mutation load or heteroplasmy is below the threshold that causes clinical symptoms, so her children may not be symptomatic, but their children could have a segregation.

For example in this case you can see that when a mutation reaches in some cases more than 60 percent then we

knew phenotypically these mutations will be expressed and cause a disease. So that allows you basically to estimate how difficult it will be for example to counsel these families and predict what the next child will be. So the mtDNA is exclusively inherited through the egg, and because of the unpredictable pattern of inheritance we've been trying to come up with a way of preventing this transmission by replacing the mutated genes in the oocytes, because a single cell will bottleneck and the genome is passed from one generation.

We've been specifically interested of course in complete replacement of mitochondrial DNA because there is a mutation, even if there is a 50 percent mutation, we didn't want any of these mutations passed through this transfer, so I'll be telling a little bit about this carryover issue. And then since it's a replacement of the entire genome, so basically any type of mitochondrial DNA mutation can be treated with one treatment. And of course it's a genetic correction, it's inheritable because it's a germ line, and of course the child who will be born to this procedure will hopefully have oocytes that don't have this mutation, so that means the next generation will be healthy as well.

So a few things that we usually consider to develop this procedure. Of course the first is feasibility

and efficacy, as I will tell a little bit later we specifically focused on metaphase two arrested oocytes. This is actually mature oocyte, from the follicle and can be re-fertilized. Another thing we do is we check the developmental potential of this egg by fertilization embryo development, and in primates of course we've done it all the way through live birth, and right now some of these monkeys are already adults. Mutated mitochondrial DNA carry over, and some of the issues we already were planning when we started these studies is nuclear mitochondrial genome compatibility.

So this is a mature oocyte, this is actually primate oocyte, and I don't know if you can see, the nuclear genome is assembled into spindles, so this is the chromosomes, it's actually a tiny spot there. In the red are the mitochondria stained with MitoTracker Red, and you can see it's a pretty even distribution. Most importantly there is no kind of clumping of the mitochondria around the spindle, which is very important in our case because we will be removing it and transplanting to another oocyte.

So when we initially started this project it was some of the technical issues of how you do this transfer. One of the problems of course is the spindle is invisible with regular microscopy because it's just a tiny chromosome in a spindle, and so we actually came up with a spindle

imaging system, the specialized microscope has been developed basically to observe the spindle proteins, and we used that for real-time micromanipulation, and this is how you would see now egg, how is the spindle actually lighting up there in that spot.

And of course we would come up with micromanipulation technique when we insert the pipette and remove the spindle with a little bit surrounding cytoplasm and a membrane. This is actually key, that you never expose the spindle to outside environment, so it always has to be safely wrapped into this membrane. And so after the procedure you will end up with the spindle and nuclear material, what we'll call a karyoplast, so it's actually a tiny egg containing nuclear material, the rest of the oocyte is also intact, and that's the only way you can maintain, as far as I know, the functional mitochondria if you want it to function in another embryo.

So this was the first step that we accomplished, and it was in 2007 when we started using this technique to isolate the spindles and to see how viable they are. And of course now we transfer them into nucleated cytoplasm taken from another female. That one also was problematic initially because normally you would place this inside of the peritoneal space, inside of the lamina lucida(?), and usually the fusion is induced by electric pulses, which is

pretty standard in most micromanipulation techniques, but it didn't work with metaphase spindles, because they respond to the selective stimulus like it's been fertilized, and they resume prematurely the meiosis, and we end up with basically already segregated chromosomes, even before fertilization, which causes a problem, and I'll show you a little bit further what it might turn into.

We started using the extract from this Sendai virus. It's actually an old forgotten technique of cell fusion, but we found that actually does not induce any negative effects or side effects, at the same time it allows you very efficiently to fuse these two couples. So in the monkey we modeled it so we would take two different types of monkeys. We have a rhesus macaque imported from Indian, and so we consider them Indian macaques, and there are Chinese macaques, and they're quite different in terms of genetics even though it's the same species, but in terms of mitochondrial genetics it's pretty drastic differences.

So we divided that to having two different cohorts of eggs, and what we would do is a reciprocal transfer of the spindles, and then we'd monitor fusion, which once we optimized everything was pretty efficient, so you would have for example 100 eggs, we would guarantee that we would produce probably at least 95 couples normally. Most importantly, if you fertilize them and the

fertilization also goes pretty normal, and we've done this with hundreds of oocytes and we don't see any reduction in fertilization or any kind of problem with fertilization.

The further the blastocyst develops, which is one of the indicators at least of normal cell develops, we'll also compare it to non-manipulated controls which was also not affected. And of course initially we would bring in stem cells, but later we did embryo transfers and we produced several infants, so the first two infants are known very well, but we totally produced I think seven infants in two different studies.

And you'll see the embryo transfer and implantation efficiencies were also not affected, at least in the Macaque model. We also tried to see if we can freeze eggs, which is very important as I'll come back to later in humans, because having two fresh egg retrievals the same day is a little bit problematic, at least in human. So with macaque what we did is we would freeze one egg, and then one would be fresh.

So after cryo-preservation of course we would thaw this egg, and then we do the spindle transfer, and this is the latest female, Crysta, who was born after the cryopreservation, after spindle transfer, and we actually did PGD at the trophectoderm to find out whether this was female, because we really wanted females. So this infant

went through a number of micromanipulations.

So there were a couple more infants that were produced, and we wanted to see right after birth, this was kind of newborn tissue, to see how much the carryover is. AS you'll remember there is a small amount of cytoplasm usually co-transferred with the spindle, which potentially can contain of course the carryover mitochondria, and we estimated that out to be around 0.5-1 percent. And in most tissues, you can see in these two females, we either wouldn't detect at all the carryover, although it would be as we'd expect under one percent in some tissues.

These two newborns were females, so we recovered the ovaries of course, and we tried to recover the oocytes. So this is immature oocyte of course, but we've been able to isolate single oocytes, and we've analyzed 12 oocytes from each of these females, and we've been actually surprised that the two of them have a considerable amount of heteroplasmy in somatic tissues.

For example in this female you wouldn't detect at all, but for example one egg had 14 percent, which still probably would be below the dangerous threshold, where the mutation has to be above 50 to 60 percent to affect, but it's still some concern. Of course these are newborn oocytes which are not mature and not really next generation yet, so we have still monkeys, they're still under age, but

we’re hoping we’ll do some IVF probably with them to see if this really will show up in F2 in the rhesus macaques.

So since these four infants were born in 2009, so now they’re almost adults. Four males actually were first born. And we noninvasively monitored their growth and development and did regular blood work, and so far they’ve been pretty healthy, we didn’t find any differences with controls produced by regular IVF.

And of course the idea was can it be done with humans, and a couple of years ago we approached our IRB requesting us to do this study, of course we said we would like to include egg donors, specifically donate their eggs for research, and then we requested also to allow us to do fertilization and study these embryos in vitro and by embryonic stem cell isolation.

It took a while to approve of this procedure because I guess we were the first to request actually our fertilization for research purposes, but we of course justified that this is needed to find out if this procedure can be in the future applied in clinical trials, so we consider it as one of the safety or efficacy studies which is done in vitro, but with normal human oocytes, embryos, and embryonic stem cells.

Embryonic stem cells are also a very important kind of addition to this in vitro culture, because as we

heard today the blastocyst would not give you that much material to study in terms of cells, it's only 100 cells or so, you cannot do a very advanced karyotyping analysis or genetic testing, so the embryonic stem cells actually allows you to do much more, so we opted to do embryonic stem cell isolation.

And so one thing we noticed, of course the spindle transfer could be done pretty efficiently, we don't get any loss of the oocytes during this procedure for lysis. However after fertilization, by counting the number of the oocytes that now have a prominent pronuclei, the percentage was very similar between controls. So the controls is basically from the same cohort of human, we would select a few oocytes and just fertilize them without any micro-manipulation and monitor their development as well.

So the fertilization rates were similar, but what we've seen is the increase in portion of abnormal fertilization, even though the abnormal fertilization does occur with regular IVF without any spindle transfer. But this was increased, and I'm going to go on in the next slide to actually show what that means. But if you separate the normally fertilized and abnormally fertilized, it seems like the development to the blastocyst was unaffected, and specifically we've been able to isolate the embryonic stem

cells lines from spindle transfer as well as healthy controls. So the abnormalities specifically were in number of pre-nuclei we could detect.

So this is a normal situation where you would see two pre-nuclei, male and female, and normally you would see two polar bodies. The first polar body is already there, but the second one will be kicked out after spindle transfer, after fertilization. WE have increased the number of zygotes that had three pre-nuclei, but they had one polar body, which was indicating that probably one of the pre-nucleus is due to the failure to separate the polar body, so due to failure to complete meiosis normally. We also had some other combinations, but these were probably in the same range as you would see in normal controls.

Of course, as I said, during normal IVF treatments the abnormal fertilization is pretty common. It’s a rule actually to do a fertilization check very early and separate any abnormal embryos from normal, and so this is what we did, and basically we’ve been able to isolate five embryonic stem cells from these 13 human ST blastocysts to spindle transfer that had normal pre-nuclei, and then we tested them by G-banding they had normal karyotypes.

So we also looked at the mitochondrial DNA carryover in embryos as well as embryonic stem cells, and

it was always one percent or below just like we would predict. As I mentioned, those three pre-nuclei embryos, some of them actually also go to blastocyst, even though the majority of them arrest. But I think we only had three pre-nuclei compared to the other combinations actually more viable, and we've been able to isolate one embryonic stem cell, and when we looked of course by karyotype analysis we could clearly see uniform triploidia across all chromosomes, and we also confirmed with other tests, and we confirmed this is due to failure to segregate the second set of maternal chromosomes, basically it's a meiosis II problem.

The healthy controls that were not manipulated, they were just fertilized, we produced nine embryonic stem cells, but we were surprised that among them we had two karyotypically abnormal embryonic stem cell lines. One had XYY, another one has XO. So this is as I said, this is normal healthy women donating their eggs for research, and they've been fertilized with standard IDF techniques, and two of them, so basically 20 percent of them karyotypically abnormal.

So the conclusions are that the entire cytoplasm containing mitochondrial DNA in human oocytes can be efficiently replaced. So of course we use mitochondrial genome from another donor, it's no recombinant, which is a

positive, and it seems like it could be applicable to any mutation type. As I showed you, ST is feasible with cryo-preserved eggs, and that seems like it's going to be part of the future clinical trials because we think that one of the problems why we have these triploid embryos is due to this failure to segregate normally a second set of chromosomes due to meiosis. We noticed this happens when we try to time two egg donations in the same day.

Sometimes we would have one retrieval early morning, another retrieval for some reason was delayed and it was late afternoon, so the first set of eggs were sitting and waiting. And eggs usually age very quickly, you have to fertilize them within a couple for hours, so waiting for 5-6 hours, that's what usually causes that, and we knew that would be a problem, and we think having cryo-preserved eggs would eliminate that problem. As I said a portion of manipulated oocytes did display abnormal fertilization.

However, we are closely now looking into the technical improvements in our ability to manipulate and avoid this, and it looks like we already have some improvements. And the normal fertilized zygotes, of course, that have two pre-nuclei and two polar bodies, we have to watch for those and separate them from others. And it seems like they aretypically normal in the neighboring stem

cells, and in terms of functioning the neighboring stem cells were also very normal.

So we were thinking about the next stage for example, who would carry on with clinical trials, basically transplanting now these embryos. So because of current efficiency, even with all these abnormalities we see it still allows you to produce at least three to four healthy embryos from each cycle, and so this would allow us to include families, in this case carriers of probably early onset of mtDNA disease, for example documented to have at least one child living or deceased, and in this case recruit healthy mtDNA donors.

As I've said, we've done this spindle transfer between unmatched human volunteers, and so far at least through embryo development and the embryonic stem cell potential, it doesn't seem like there's any unmatched within population of humans that can cause any abnormalities we could detect. And of course we could do PDD, we could probably biopsy trophectoderm, pre-implantation development, can confirm one more time the normal of the karyotype and probably any carryover, and then follow up with the birth and development of healthy children. So that's basically all, and I would be happy to answer any of your questions.

DR. SNYDER: We are open for questions.

DR. GEARHART: A couple of questions, if I may. In your fertilization slide you said that the outcome is the same or close to what it generally is in IVF. Was that one of the comments you made, in other words with less than 50 percent --

DR. MITALIPOV: In the Rhesus macaque we never saw abnormal fertilization. So when we used the same protocols to humans, without any modifications, that's when we've seen these abnormalities.

DR. GEARHART: The second question, can you get around the use of Sendai virus? Do you have a way, if you carry this into human embryos there could be an issue, right?

DR. MITALIPOV: It's not the live virus, it's an extract. It contains the envelope without any viral RNA. But we tried to go around it. So soliterperation(?) didn't work, polyethylene glycol didn't work, so it has lots of side effects, it's very toxic. So it still seems like the Sendai extract is the best option. We don't have anything better than Sendai.

DR. GEARHART: The last question if I may is obviously when your work follows on published work that may have a more negative outcome, you are forced to answer why is it so different. And what obviously I'm referring to is the publication in 2000-2001 and later of the three parent

embryos, in which we know that out of the 17 that were first looked at there were two with Turner's, there was another with intellectual disability, and so there is something that precedes you and puts you in a bit of a hole. And I realize this is a very different contrasting situation, but how do you respond to that? I'm sure you've looked into this carefully, and what kind of a response would you have?

DR. MITALIPOV: In those clinical applications of this mitochondrial transfer, even those were not done using this spindle transfer procedure. I bet they were done by either cytoplasmic injection with ICSI, which by itself cannot basically replace mitochondria. so you could only inject maybe five percent max, but when you're injecting you're actually injecting lots of fluid, and when you do XC which is a bad thing, because you can induce premature activation of the spindle, and that's what you will see.

So the chromosomal abnormalities are very dependent on how you introduce that additional cytoplasmic mitochondria. I think some of the earlier studies as I remember were done by transfusion. So they're transfusing that additional cytoplast. And we know for sure that it doesn't really work very well, because this induces the premature activation of the spindle. So I think if we avoid that that's why Sendai is a key, even with all these

additional concerns that you could have some viral contaminants, but so far it's the best solution to avoid any abnormalities.

DR. KEEFE: As you move into humans, the dictum of primum non nocere, first no harm, takes special importance. And so just a cautionary tale and then a question. The concern we heard earlier about redox and long-term effects are troubling in that the animals in which they were studied, the ruminants, were not only related to culture, they were also related to the transfer itself, because even if they cultured them in the fallopian tube they had the same effect. They found that in fact the development in vitro of these animals that went on to have problems were better looking oftentimes in terms of morphology than those that didn't. So they had larger inter-cell masses, they developed to blastocysts earlier, so that's a little bit tricky.

And then the second point that you made about the mutations that you looked at, they were actually neutral variants rather than disease causing mutations, the cutoff of being 50 percent of course as you know is not necessarily specific to all diseases. The most virulent of these, for example the MELAS, there are studies that in some pedigrees 30 percent heteroplasmy, 70 percent offspring have been reported. So there is enormous

variation, and Dr. Dimauro could tell us more about this, but huge variation when you're dealing with these virulent strains in terms of the percent. Those are just two cautionary points.

And then the question is, one of the concerns, getting back to the concern of the burden of proof being on us to show safety, is that the animal born from this, it may be predisposed to metabolic problems, may not necessarily be revealed in the laboratory setting, the question of generalizability.

What kind of diet did you have these monkeys on? Did you give them kind of the McDonalds supersize stress test? In other words, have they been exposed to big macs and so on to see if it covers something? The goal here is to look for a problem, not to not find it, because in the end it always comes out, and we all want to find that out before. So I'm just wondering about the diet and what kind of metabolic studies you've done.

DR. MITALIPOV: So in the Rhesus monkey we haven't done any invasive monitoring them yet. These five monkeys are still alive. I think we want to breed them, and these four males are adults this year, so they plan for breeding. But we're doing a little bit more in the mouse.

As Key(?) said there are always advantages and disadvantages of each model. With monkeys it's a long

development, and of course the cost associated, we couldn't produce that many, it was all controls to study, but in the mouse of course we could produce hundreds, and both spindle transfer and pronuclear transfer work very well, and so we're looking into this by basically stressing them out and to see if we can actually find some differences in mismatch.

Speaking back to whether this mismatch, even from healthy haplotypes, will cause some kind of problem. Since we don't have any evidence, credible evidence, to basically match, I guess we could propose matching mitochondrial haplotype of the patient with a same haplotype of very close, which is no big deal, it can be done pretty quickly if that seems to be an issue.

So the first question I guess was about whether there are differences in embryo development. So far in most of our experiences, of course in humans it was healthy volunteers, and we didn't see much of a difference in terms of embryo development of blastocyst development. Neither in the monkeys, the monkeys were also healthy. Unfortunately we don't have the mitochondria disease model in the monkey.

DR. CEDARS: So I have several questions as well. First, in terms of the primates that you've looked at, one of the concerns I have would be the potential impact or stability of the mitochondria to the normal effects of

aging on mitochondria, and are your primates old enough and have you specifically looked at the functionality of the mitochondria to see if these offspring might be at some kind of increased aging risk, or the sort of impacts of aging because of their mitochondria.

DR. MITALIPOV: So far they have been juvenile. So they turn to be adults after about five or six years, but I think they age at 20, so we still have a few years to go before we see if there is any premature aging in them. The plans are, of course we will follow them up as long as we can, as long as NIH is funding this research.

DR. CEDARS: But is there a way, like David was suggesting somehow to stress them or try to see what the capacity is for mitochondrial health.

DR. MITALIPOV: We have some tests, we could put them in treadmills, those are plans in the future. As I said I had to put two RO1s together to produce the seven infants, quite an expensive project. Both of my RO1s are up for renewal, and I am requesting that I will do all these tests, I will see what they say.

DR. CEDARS: And then the other questions I had are about the clinical trial that you're proposing, and obviously some of these are going to be questions that we're going to discuss. But I had two questions. When you're talking about the people that you include in the

trial, given the discussion earlier and the chances for an unaffected low-load offspring, even in a woman who's affected, how do you assess that your therapy is actually successful relative to what the person may have done on their own when they could have had a healthy child?

DR. MITALIPOV: That is what we are proposing to have families where they at least have one child affected, and that means there is a likelihood that the next child they would conceive naturally would have the same mutation, because we have no way to know it. But one thing we would do for example, if we recovered 10-15 eggs from that patient, we'd just take the spindle and we'd transplant the healthiest cytoplasm. But remaining cytoplasm from this patient, each will be analyzed separately, and we can predict for sure what mutation was in each of those eggs. So hopefully this will come in clinical trials.

DR. CEDARS: And then the other question I had, you talked about doing PGD, and again this issue came up, is how reflective is the trophoblast of the inner cell mass.

DR. MITALIPOV: We have done in the rhesus macaque studies, at least in terms of heteroplasmy, there is a segregation in blastomeres at the cleavage stage, so maybe that's not predictive. But once you sample trophectoderm, which normally could do at least five-ten cells, so it

could be more predictive, and it looks like there is a similarity between for example the heteroplasmy level in IC un-separated(?) versus trophectoderm, at least based on primate studies.

DR. CEDARS: So without being too provocative, if you feel like you can test it with a trophoblast biopsy and a certain subset of the embryos will be normal, couldn't you just do pre-implantation genetic diagnosis and not manipulate an oocyte and find the unaffected embryos to transfer?

DR. MITALIPOV: So far there has been a very mixed view about using PHD for mtDNA mutations. There have been very view children born, at least we have a data about one that has been born just a couple years ago with MELAS, and even though during pre-implantation sampling I think it was measured around 10-15 percent, but the child after birth had more than 50 percent.

DR. CEDARS: That is why I asked the question.

DR. MITALIPOV: I think mostly we are proposing to do some sampling of trophectoderm, not maybe as much as to predict the mutation load, because it's going to be below one percent anyway, but to see if there are chromosomal abnormalities, because we've seen some of these diploid in the embryos, that's why I think with the CGH we can clearly pick up if there are some chromosomal matter in that

embryo, so we would avoid transplanting that.

DR. BUGBEE: If there is one thing I learned today, there are a lot of things that are hard to measure about mitochondria. So with that in mind I think you've partially answered the question. Can you or anyone else define what a healthy mitochondrial egg donor would be, and how you would measure that person or define them.

DR. MITALIPOV: I guess there are certain criteria for selecting egg donors. A thousand thousand babies are born through egg donations. So our criteria for selecting egg donors or cytoplasmic donors would be the same. But of course we could look into family history to see if there has been any family history in mitochondrial disease. But other than that we don't have any specific indication to look into mitochondrial genome in the eggs. Once you have it I think you have to basically waste an egg to analyze maybe coding number mutation. So at least I don't have any kind of clear plan how to select egg donors in terms of mitochondrial disease.

DR. BUGBEE: Do you think it would be different in this setting versus egg donors?

DR. MITALIPOV: I would say it would probably be similar. For example, when we recruited these women for our study, many of them actually were egg donors for reproductive services, so that means they'd been already

probably prior tested through normal development.

BUDBEE: So you wouldn’t suggest any different screening.

DR. MITALIPOV: Not unless you guys decide. We can do whatever you say.

DR. BUSTILLO: I have a question, back to the virus issue. You cannot do an ICSI-type type procedure and insert the spindle into the cytoplasm?

DR. MITALIPOV: Just by direct injections? No, it just doesn’t work. I actually don’t know how you could isolate mitochondria separately. Spindle is the same, it’s a very fragile system and you have to handle it like with the gloves, very gently, and some of the problems we’ve seen with fertilization because of this may be rough handling.

DR. ROSE: So I am confused. We’ve heard about interactions between the mitochondrial DNA and the nuclear DNA, and you have up there recruit healthy mitochondrial DNA egg donors. Are you talking about doing full mitochondrial genome sequencing in order to determine there are no mutations in any, i.e. you’re looking for homeoplasty?

DR. MITALIPOV: No I think if we are doing donor kind of genetic testing, mainly to trigger out what the haplotype is, I don’t think we can detect what mutation. I

mean, women may not have mutations in her somatic cells in the blood, but it could be in the eggs, so it would never be predicted. But at least we could do it pretty easily because the mitochondrial genome is not that big. We could sequence it and tell exactly what match or mismatch would be between donor and patient. That's the way we could select the donors at least in terms of matching. Probably if there is indication that these haplotypes or haplogroups is not compatible with that, within humans, then this would select something that's compatible.

GOLDMAN: to get back to the trial design issue, can you be more specific in terms of what your disease targets are here? Early mitochondrial presentations, Leigh's or MELAS, even amongst these the sibling penetrance is so variable that I think I would be concerned in terms of how one would set a trial up. So if you could be more specific in terms of exactly what your disease targets are.

DR. MITALIPOV: I think what I meant is we would choose specific mutations that cause these symptoms early in their life, there could be this Leigh's disease where most of the children usually die in the early childhood. It probably wouldn't be wise to choose for example Leber LHON, because sometimes the blindness occurs in teens, we would have to wait 15 years to document in the normal health. So that's why we thought that we will choose specific

mutations/disease types with early onset so you could see within maybe three to five years after birth the lack of symptoms.

GOLDMAN: So what would the required power be? I mean given the variability in disease presentation, how many patients would have to be done before you'd have some sense that this was a successful manipulation.

DR. MITALIPOV: At least in the early phases probably, because this is actually a rare disease, so we would choose around five to ten families for the first phases of the trials.

DR. COUTURE: So in contemplating a clinical trial the question is to ask what it is that's being tested, and I think there are at least a couple of things that would be tested here, the first of which is whether heteroplasmy would manifest itself in F1s, kids.

DR. MITALIPOV: Immediately after birth, of course.

DR. COUTURE: The second question is whether or not heteroplasmy, whether it manifests in disease or not, would be transmitted to F2s. This is another big issues. As far as I know your studies haven't yet addressed that, that's what you want to do next in the primates. For that reason, one of the questions that's going to come up is are you contemplating limiting these initial trials to male

offspring to prevent the second part of that, and just address the safety issue up front, which is typically what initial clinical trials are structured around. Not efficacy, it's structured around safety.

DR. MITALIPOV: It is a possibility. We can clearly select the for male, again through trophectoderm biopsy we can clearly tell whether this embryo is male or female and transplant only male ones, so that way at least we would avoid if there is any issue with F2 generation. But we would never figure out then whether there is any or not if we never produce female offspring.

DR. COUTURE: I guess I am kind of thinking, I'm not proposing or advocating one approach or the other, but one approach to getting clinical trials started perhaps sooner would be to limit the safety concerns and let the animal studies catch up. That obviously raises issues that we're not to discuss today, whether you should be gender biasing offspring and that sort of thing.

DR. SNYDER: Let me emphasize that clinical trial design is doing to be the topic of this entire afternoon and into tomorrow morning. Right now we probably should limit to asking Dr. Mitalipov things to clarify what his intent are and his data, and then we'll have plenty of time to discuss this. Are there any other questions specifically about the presentation?

I guess I had one last question. In the humenia(?) cells that you've generated, have you looked at mitochondrial function in those, and then the second, have you applied this yet to any oocytes that already have a mitochondrial mutation, or only normal mitochondria.

DR. MITALIPOV: The short answer to both of them is no. So we haven't looked at any mitochondrial function in these STE cells, but I believe the presentation did after me addressed some of this. No, we haven't been able to recruit, at least we tried patients, a woman with mitochondrial DNA mutations. A few families have been interested that there be more interest in clinical trials rather than donating oocytes for research.

DR. SNYDER: Thank you very much, and I think we will move on to the last speaker before lunch. And that will be Dr. Egli addressing efficacy and safety of nuclear genome transfer in human oocytes.

Agenda Item: Addressing Efficacy and Safety of Nuclear Genome Transfer in Human Oocytes

DR. EGLI: Thank you very much. I am grateful for having the guidance of this committee and of the US government, how to best translate this technique into a therapy. I am going to speak exclusively about using this technique for the prevention of the transmission of mitochondrial disease. And this is just a brief overview

that by now you may be familiar with. The nuclear genome of one oocyte is removed and transferred into a enucleated oocyte with a different mitochondrial genotype. And so the FDA has put forward a few clinical trial considerations, and I'm going to address these point by point in the way our results speak to those considerations.

So first, the question is whether there is the chance that mitochondrial disease might reemerge. The answer according to our results, as you will see, is that the carryover is extremely low, and what we've found is a drift to homoplasmy. The expectation that we have is very strongly that a child would not have mitochondrial disease.

So here is the human oocyte stained for mitochondria on the left, and at the greater magnification on the right, the nuclear genome that has been removed. And what you can easily see, there is no enrichment of mitochondria around that spindle, but there is a little bit of mitochondria taken out with the nuclear genome with some of that cytoplasm.

And so to determine how much that will be we did the following experiment. We first measured the size of those karyoplasts that are removed. An because the spindle size is defined, it's between 18 and 20 micrometers, the size of the karyoplast can be reliably of the same size. And so what we find is that the karyoplast size is about

200 times smaller in volume than the egg. And then we measured the mitochondrial DNA content in those eggs, and it's also about 200 times less than what's contained in the oocyte.

So what we expect is a carryover of about 0.5 percent. And when we looked to test whether our expectations hold true, we looked at the pre-implantation stage. And so you can see here at the cleavage stage, at the morula blastocyst stage, we looked at 24 of those transfers at that stage. They were all below 1.0 percent, and the average below the 0.5 percent mark that we expected. We also derived pluripotent cells from these after pre-implantation development.

Now just a very short overview of what these pluripotent cells are. We called them swaPS cells because they had their nuclear genome swapped into a different egg. And so these pluripotent cells, they are derived from the blastocyst stage, they grow indefinitely in culture, they can give rise to all cell types of the developed body, and for the cells we determined the population doublings, and it was about 31 to 33 hours. Cells are passaged about twice a week, and so if you calculate, within two months you would have about 45 population doublings, and if you were to keep all cells that would make about 3.5x10^13 cells, and that approximates the number of cells in the human

body.

But we didn’t just do that, we cultured them for over a year. So not just for two months, to just determine whether that old mitochondrial genotype ever comes back, and it didn’t. You can see here in the beginning when these stem cells were derived the mitochondrial heteroplasmy was very low, but it first decreased, and these levels are essentially at undetectable levels using the techniques used.

We also differentiated them into neurons, beta cells, cardiomyocytes, and did not find that that mitochondrial genotype reemerged. We also sent them through a bottleneck using reprogramming technologies. And so there was one outlier, one of 28 had higher than one percent heteroplasmy, it went up to three percent, but then again it decreased within a few passages to essentially zero, to undetectable. So what we conclude from this, heteroplasmy is expected to be extremely low, and there is a drift to homoplasmy.

What about disorders due to nuclear mitochondrial incompatibility? The experiment we did to address that was the following. We used parthenogenesis instead of fertilization to address this point. And we did that because if you transfer one genome from one egg to another and any damage is introduced, then it should be revealed,

because development and mitochondrial function depend entirely on the interaction between the transfer genome and the new mitochondrial genotype.

If there is also a sperm genome it may compliment for any of these defects that the manipulation might introduce. So in many ways this parthenogenetic experiment, even though it’s not useful for therapy or for reproduction, it’s extremely useful to address these scientific questions.

And what we did, we sequenced the mitochondrial genotypes of two donors and used in this particular experiment, and they had a different mitochondrial DNA haplotype. So Dr. Shadel this morning said we don’t know the consequence of what happens if two different mitochondrial DNA haplotypes are mixed in the egg.

So here’s the experiment. These mitochondrial DNA haplotypes not only had a different in haplotype, they also had one polymorphism with unknown function in the TRNA veiling gene, it’s a mitochondrial gene. So the outcome from this experiment is really interesting. What you see is the implantation development of these eggs, again of the parthenogenesis activation, up here would be the manipulation, polar body extrusion, one cell stage four, eight, morula, and blastocyst.

And you can see some of these blastocysts have

really good morphology. That would be the stem cell line derived from that blastocyst here. Parthenogenesis not quite as efficient as in vitro fertilization. So we take a little hit with this experimentalization in the efficiency of blastocyst development. This would be IVF, it varies by about 50 percent, you would be pathogenesis without any manipulation around 33 percent. But the manipulation itself did not reduce that efficiency.

Likewise, the derivation of stem cell lines was no different, and stem cell line derivision efficiencies is an indicator of blastocyst quality. The stem cell lines that may stay variable, they behave like any other stem cell lines, they express the right pluripotency markers, they differentiate into cell types of all three germ layers, and the wonderful thing about the stem cell is you can differentiate them into the same cell type that an adult human being has essentially.

So in this case what we did is we obtained, Dr. Sauer took also skin biopsies from the oocyte donors, and we differentiated the stem cells into skin. So what you see on this picture is a primary skin cell from the oocyte donor, and here you see the skin cell with the same nuclear genome, but after that swap. So these two cells, they are very similar in their cell type, in their differentiation state. They essentially share the same nuclear alleles

here, but they have different mitochondrial DNA haplotypes. So what's the consequence of that on function of mitochondria?

And this is the end result. This is the skin of the oocyte donor who donated the mitochondria. So oxygen consumption rate, which would be respiratory activity of mitochondria. This is the skin cell from one egg donor, this is the skin cell from another egg donor, and these are two cell lines that contain the nuclear genome of this egg donor, but the mitochondria of this egg donor. And you can see here that these mitochondria work just as well with this nuclear genotype as they did with here. There is no difference between this one and these two. So our experiment does not support the notion of incompatibility.

And then we looked at mitochondrial enzyme activities in these cells, either in undifferentiated EA cells, or in differentiated fibroblasts. Again we did not see differences in the activity of mitochondrial complex enzymes. So the conclusion is that cells with the same nuclear alleles but with different mitochondrial genotypes do not show a difference in mitochondrial function, at least in this particular experiment.

This result is not entirely surprising, since you would expect that from human production. Although the mitochondrial genotype of a woman is obviously inherited

with half of her nuclear alleles, these nuclear alleles of a woman can combine with a different mitochondrial genotype by inheritance through a son. So in many ways when I listen to discussion I sometimes feel we are talking about the risk of combining different alleles that are just simply inherent to human production.

The third point is so what about epigenetic modifications that may result from this technology. Epigenetic modifications may affect the rate of pre-implantation development or the differentiation of those cells. They could also affect gene expression, and so we tested for that. Again, as I pointed out, development was no less efficient after this manipulation. The stem cells that we made could differentiate into the cell types as any other stem cell line would.

And when we analyzed the gene expression profile of those cells we found that they clustered together with un-manipulated cells, these are un-manipulated parthenogenetic ESL cells, these are the swap cell lines, this is another un-manipulated cell line, we found only one gene that was differentially expressed between the two groups, the manipulated and the un-manipulated ones, and this is probably related to variation in culture technology. ESLs cells are very prone to spontaneously differentiate, and so there is a great variation within

such an experiment as well.

So the conclusion from that is our experiments, to the extent we were able to address that, they do not support the notion of epigenetic changes. I think it's important to separate the topic of oocyte nuclear genome transfer from the topic of somatic cell nuclear transfer, where there is very strong and clear evidence for epigenetic changes. I think in many cases we cannot use literature that uses somatic cell nuclear transfer in this context.

What about potential for birth defects and disorders that are associated with mitochondrial manipulation? So we first looked for copy number variations, we also looked at karyotypes, and we found the following. So, stem cells contain normal karyotypes, as you can see here, 46XX, and when we looked using ray experiments at the copy number variations, these are the number of copy variations in the germ line that are simply naturally induced, and there are a few, and the manipulated lines don't have an increase in copy number variations.

So that's assuring that the manipulation doesn't break the nuclear DNA and then somehow incorrectly seal it back together. But what we found is that in some cases, although in many cases the second polar body extrusion was normal, in some cases we found a gain of chromosome as

showed here, and that indicates this heterozygosity here, these are SNP arrays, Single Nucleotype Polymorphisms arrays. If the value is one the polar body was extruded normally. If the value is not below one, it wasn't. And so here this additional chromosome was not extruded in the second polar body, and here this heterozygosity indicated that it happened at that stage.

And so it is possible, we think that this relates to this issue that Dr. Mitalipov also referred to as premature spontaneous activation of human oocytes. He found that this has something to do with the way the nuclear genome is transferred, and also the state of the microtubules in the nuclear genome. And so what this here shows is the spontaneous activation rate, so the exit from meiosis, and as Dr. Mitalipov said, if Sendai virus is used we did not see the spontaneous activation. However if electro-fusion was used there was spontaneous activation.

We found though however if the karyoplast was cryopreserved before, or cooled, this spontaneous activation again did not occur. Polar body extrusion occurred quite efficiently, but as you see here in some conditions it did not always occur. So I think if I have any concern about this technology I think it is about the karyotype of the eggs that are being generated, and I think it's worth to have a look at this.

I don’t consider this necessarily a showstopper, because there are situations in RDF treatment like advanced maternal age where the karyotypes abnormalities are increased, and yet these women are eligible for treatment. Also, with improved techniques, as we have shown you that some of these techniques show a much lower rate of the spontaneous activation, and they may in fact eliminate this issue altogether. It is possible that we will add to that with more numbers, because the numbers have been quite low.

So what about potential toxicities of reagents used in this transfer? The exposure to the reagents is very short, it’s approximately five minutes for one agent called Cytochalasin B, this is an agent that is used to make the egg more like an oil droplet so that genome can be removed quite readily without breaking the egg. And after that reagent is used it is exceedingly diluted. So you can see here in this manipulation, exposure to Cytochalasin B is for about five minutes, fusion takes also a few minutes, certainly less than 30 minutes.

So the way this is done is an egg is transferred into a well like this, and this well could contain something like 500-750 microliters of medium. During the exposure the egg is exposed to five microgram per ML Cytochalasin B. Cytochalasin B depolymerizes the actin cytoskeleton. Then it’s washed through about four drops,

and so each dilution, the carryover from one drop to the other is very low, and each watch would be about 1000-fold of dilution.

And you can see in these slides, this is the only experiment I show you in mouse eggs, and I did that specifically for you actually yesterday. This is a mouse egg that had been treated with Cytochalasin B, and this is the actin cytoskeleton. It's 90 minutes after treatment and washout, and you see that polar body extrusion is occurring, that the actin cytoskeleton correctly cleaves off that polar body from the main egg, only if you keep Cytochalasin B on actin cytoskeleton is morally localized to periphery. So it's important to know that this compound, Cytochalasin B, is very reversible, and there is very good developmental outcomes in animal model systems. In fact in monkeys and mice already in the 1980s these compounds had been used for this purpose.

So with this I would like to thank the people who have contributed to the work I just showed you. I enjoyed a very longstanding collaboration with Dr. Mark Sauer of CWSC(?), much of the work has been done with Daniel Paul, Nathan Jeff, he's an expert in karyotyping of human embryos, and I would also like to thank Susan Solomon for her continuous support of this type of research.

And then if you allow me may I just conclude with

her greetings that she would like to convey. She writes that unfortunately she couldn't join us personally but she writes: Dear Chairman Snyder and members of the Cell, Tissue, and Gene Therapies Advisory Committee, thank you for inviting Dr. Egli to discuss the technique he developed in the NYSCF laboratory that may prevent inheritance of mitochondrial diseases in children.

I applaud the FDA for focusing on this emerging area of unmet need. While the scientific and regulatory conversation about how to advance this science is critical, the promise this research holds for the one in 10,000 people affected by mitochondrial disorders and their families cannot be underestimated. Women who carry such mutations often have to choose between not having children or undergoing in vitro fertilization with donor eggs in order to avoid passing on the genetic condition. For these women, their children, and the many who may be affected in the future, Dr. Egli's work holds the promise of preventing inheritance of these disorders. It's exciting for the US and the New York Stem Cell Foundation Research Institute to be on the forefront of this important field. Sincerely, Susan Solomon. Thank you.

DR. SNYDER: Thank you. We're open to questions.

DR. WITTEN: Thank you for that talk, and also for looking so carefully at our document that we posted on the

web with our discussion topics. I just want to mention again that the topic for tomorrow afternoon on FDA clinical trial considerations is separate from what we're talking about here. We haven't made any decision about whether clinical trials of this technology if they're proposed would be allowed to proceed, what we have on our document that's on the web and that you all received are discussion topics, and we're looking forward to hearing the scientific input we get from the committee. So I just want to make sure it's clear that those topics are not the same topic.

DR. SNYDER: Thank you Celia.

DR. CEDARS: I also want to thank you for that presentation. I had one question, because obviously you always start with sort of the people with the best potential, so these are young, healthy egg donors. And if you're seeing karyotypic abnormalities there, is there a concern as women get older, because whether these are women for mitochondrial disease, and certainly in the case of women who have age related infertility, is the instability of the spindle and the arrangement of the spindle going to put those women at even greater risk with these technologies for aneuploidy?

DR. EGLI: So what I can say as I showed you we find evidence for these karyotype abnormalities. This does not necessarily mean that they are greater. The numbers we

have checked so far are rather low, many are normal, some are abnormal. That's also true for human pre-implantation development. So I think an outstanding question for us to solve is is there any increase. I think if there is any it's probably low.

And yes, we only used oocytes from women in their 20s and early 30s, and I believe that would be the target population for a clinical trial. These are exactly the eggs you would be using on the donor side, just as is being done for treatment of infertility today for donor eggs. Again, I would like to point out this topic I presented to you is not for the treatment of infertility, it is entirely and only related to whether this would be efficacious for presenting the transmission of mitochondrial disease.

DR. KEEFE: It was reassuring that the amount of heteroplasmy was very low in culture, the pluripotent stem cells tended to extinguish. But I'm wondering if you didn't use a neutral mitochondrial DNA variation but one such as the so called common deletion, the 5000 base pair deletion where in many settings it has a replicative advantage because it's only two thirds the size, and it's been shown to preferentially replicate. Has anybody made an IPS from a patient with hereditary optic myopathy so you can see if the kinetics of the mitochondrial DNA in pluripotent stem cells is similarly reassuring.

And then of course the collar of that would be unless you have that, perhaps as you think about a disease to pick you might not want to pick the one that tends to replicate itself. And I know in certain tissues the pronuclear location of the mitochondrial DNA further confers replicative advantage, so that even if they start as a small number the fact that they're shorter and they're coming in in a position that's favorable to the nuclear encoded transcription and replication factors, would you want to say start with MELAS and stay away from the common deletion?

DR. EGLI: I think part the question was answered by Dr. Dimauro. These deletions are very rarely present in the egg. So this is mostly about point mutations, is that correct? So I don't think that the question is immediately relevant to these mitochondrial disorders and this technique.

DR. GEARHART: A couple of questions. It's a lot of work that you've done, I appreciate you trying to address some of the issues. 400 trillion cells in a dish is a little bit different than 400 trillion cells in an embryo, and the pathway it's taken, the selection that occurs with respect to the nucleus and the mitochondria, particularly when we learn that certain types of mutations are favored in certain types of cell types. A little bit of

caution with some of this.

DR. EGLI: Thank you for that point. I couldn’t agree more. This is an in vitro experiment. We just took it as far as we possibly could, and to the extent that our results speak to the points being considered that’s what I’m presenting.

DR. GEARHART: The issue is that to me it is limited in a dish versus an organismal evaluation, which you can’t do. And doing two combinations from healthy patients and looking at whether or not a nuclear genome and a mitochondrial genome are going to be compatible, therein to me lies a little bit of the limitations of your studies, that’s all.

DR. EGLI: Yes, I think that is again an important point. As I pointed out, the combination between new nuclear alleles and mitochondrial alleles is recombinations happen during human reproduction, and I think for additional evidence regarding this topic we should also be looking at that. What’s the evidence in human reproduction that there is such incompatibility.

DR. SNYDER: Are there any other comment or questions from anybody on the committee? Okay, I guess we’re ready to take a lunch break. Please be back here at 1:45 sharp so we can begin again and stay on time.

(Where a luncheon recess was taken at 12:30 p.m.)

A F T E R N O O N S E S S I O N (1:45 p.m.)

DR. SNYDER: So we have one last presentation prior to the open public hearing and prior to our discussion later in the afternoon. And that's from Mary Herbert, and her topic will be the potential of new reproductive technologies in reducing the risk of mitochondrial DNA disease.

Agenda Item: Potential of new reproductive technologies in reducing the risk of mitochondrial DNA disease

DR. HERBERT: Thank you for the invitation to speak. I was asked to talk about the new techniques, the rationale for our methodology, the ongoing work and additional experiments that I think might be required, and ideas for clinical studies. And I've also put in a section on what is currently available for these women who are affected by mitochondrial DNA mutations, and also in the end I'm going to just give an indication of where we're at in the UK.

You'll have seen this already, that the mitochondria we get in the fertilized egg come entirely from the mother, and then on day one we've got a zygote, with the maternal and paternal genome, separately packaged

instructions called pronuclei. And then over the subsequent days the one cell embryo divides to four cell, eight cell, and then by day five to six we get a blastocyst, which on average contains somewhere between 60 and 200 cells.

The point is that by the time you get down here, you start with this population of mitochondria from the egg, and that partitions between cells during division, and by the time we get down here we have very few cells that give rise to the baby, with relatively few mitochondria in each. And that becomes important when you're dealing with a mitochondria DNA mutation, because mutations of mitochondria may segregate unequally by the time you get down here, giving tissue specific effects and reproductive effects that we've already heard about.

Now, the interesting thing, before I move on from this slide, is that these women with mitochondrial DNA mutations, my colleagues who are mitochondrial geneticists tell me that there is no evidence of infertility in these women, so I think that's an interesting point in relation to the impact of mitochondrial function on development at the early stages.

So the segregation of mutated mitochondrial DNA during development gives rise to eggs with varying degrees of mutation. When a woman has a mix of mutated and wild type mitochondrial DNA her eggs will contain varying

levels. And that becomes very difficult in terms of reproductive choice, because it is impossible to know whether she will have an affected child or not.

So the options that are currently available, earlier this morning we had a comment about egg donation, and of course egg donation is available, but egg donation is something that women come to generally in the process of accepting their own infertility, and these women are not infertile, and from what I hear from my colleagues egg donation is not an option that they favor.

So we can offer pre-implantation genetic diagnosis. These programs are at a very early stage of development, I know of three centers in Europe, including ours, that are offering this treatment. So pre-implantation genetic diagnosis, for those of you that don't know, it's a well-established technique for preventing transmission of nuclear genetic defects. It involves IVF treatment to generate multiple embryos, and then removal of either one or two cells from a 6-8 cell embryo, which is then sent for genetic analysis.

So we can also apply this to look at the mitochondrial genome. We can apply it to help us distinguish between embryos with low mutation lows and high mutation loads. So our procedure is just to remove one cell, send it off for analysis, and select those with the

lowest mutation load for replacement.

And then so a question related to this technique is does the mutation load of the biopsied cell represent the entire embryo. We are gathering evidence on this along with the other centers in Europe offering this treatment, and the evidence so far indicates that differential segregation of mutations between blastomeres is not a problem up to the eight cell stage at least.

We've also done some analysts of blastocysts, and the trophectoderm does seem to be representative of the inner cell mass. However, the major point about PDD is that it is not useful in cases where a woman is homoplasmic for the mitochondrial DNA mutation, or if she carries a high mutation load. In those cases you will get embryos in this end of the spectrum. So PDD is good for if we have a woman who is going to produce embryos with low mutation loads, but for high mutation loads it is not useful.

So the alternative strategies, which for the purpose of this meeting is to say can we uncouple the inheritance of nuclear DNA from mitochondrial DNA. And one idea would be to move the mitochondria, but obviously that's not feasible, so the idea is to transplant the nuclear DNA. And in principle this can be done either before or after fertilization. And you heard from Dr. Egli and Dr. Mitalipov about using the unfertilized egg and

transferring the meiotic spindle, and for that you've got to use a polarized light in order to see the spindle. Or the other option is to use the pro-nucleus stage egg, the fertilized egg.

A clear advantage here is that you can see what you're dealing with by light microscopy. This is the one that we opted for on the basis of that, firstly so that we could see what we were doing, and also there had been extensive experimentation in mice to show that these procedures seem to be without harmful effects throughout generations of mice. And also a Japanese group then generated a mouse with a mitochondrial DNA deletion and found that they could generate embryos in which activation was not present in the embryo.

So in terms of clinical treatment the concept would be with pronuclear transfer is that you take a donor egg from a healthy donor, and you take an egg from an infected woman, you fertilize them, you generate pronuclear stage eggs, you enucleate both, and then you reconstitute the pronuclei of the affected woman with the cytoplast of the health donor, and that enables this woman to have her genetically related child while greatly reducing the risk of transmission of disease. And the comparison then of course with the fertilized egg, with using the unfertilized egg, is that you just transfer the spindle and do the

fertilization after reconstitution. Then after these procedures obviously we want to see embryo development normally to two, four, eight, 16 cell blastocyst.

So this is the sort of scheme we're looking at. And our question when we started out was whether pronuclear transfer is technically feasible in human, and that was quite an important question because in human these are really quite big structures, ranging from 25 to 30 microns each, and then of course we wanted to know can we minimize the level of mitochondrial DNA carryover, and then can these reconstituted zygotes develop to the blastocyst stage.

Starting out on this we did not have a ready supply of material to work with because these normally fertilized eggs, women want to keep them for their own treatment. So we did proof of concept work using abnormally fertilized, either with one pronucleus or three pronuclei. And having gone through a great deal of regulatory hoops to get permission to do this in the first place, we started out wondering if we could safely remove them without too much damage to the embryo.

That involved depolymerizing the cytoskeleton, which was depolymerizing the actin and microtubule networks. That differs from spindle transfer in that you only have to inhibit the actin network for spindle

transfer, not the microtubules. And obviously, as Dr. Egli mentioned earlier, with the reagents we used to inhibit the cytoskeletons we have to do tests for reversibility and cytotoxicity.

And then in the fusion step we tried electrical fusion. And there's a problem with lysis in human eggs and zygotes, in that they don't tolerate electrical fusion very well, so we also went to viral envelope protein from inactivated Sendai virus, and using this we can get high rates of fusion without lysis. In doing this with abnormally fertilized zygotes we found that un-manipulated controls developed to blastocysts at a rate of 17 percent, which is low compared with normally fertilized, but that's the material we were working with.

And then with our pronuclear transfer procedure this was in the range of eight percent. So we got some loss of viability due to the procedure, or potentially due to mixtures of –- in the humans it's not easy to distinguish the male from the female pronucleus, so that might have been a cause of the reduced viability as well. And importantly we got less than two percent mitochondrial DNA carryover.

So on the basis of that sort of proof of concept work, we thought this is worth investigating further. And our ongoing work on this is we're looking at the effect of

pronuclear transfer on embryo development of normally fertilized eggs, and so we've been through all the regulatory hurdles and processes about being able to use normally fertilized eggs for this.

Our aim with those is to maximize the yield of high quality blastocysts, and I'll get onto this more in the next slide. Then we want to test for normality using a range of parameters to compare pronuclear transfer with control blastocysts generated by XC, and then we want to look at the fate of the karyoplast associated mitochondrial DNA. We're also carrying out in vitro studies to compare pronuclear transfer and spindle transfer.

I'm sorry I can't speak a lot about our ongoing work, because it's not published. But I was asked to speak about the additional in vitro studies to test the likely efficacy. Coming from sort of a clinical background, I suppose it's a question of are women likely to get pregnant from this procedure is question number one. So the blastocyst contains several different compartments. We've got the outer layer, the trophectoderm, which forms the placenta, we've got the inner cell mass which consists of primitive endoderm and epiblast cells, primitive endoderm becomes the yolk sack, the epiblast can give rise to fetal tissues or to embryonic stem cells if it's X-mounted and cultured in vitro.

The establishment of a viable pregnancy requires the trophectoderm, the primitive endoderm, and the epiblast, whereas the establishment of ES cell lines requires only epiblast. So the point is in the evidence we've seen so far from either pronuclear transfer or spindle transfer, we've seen not very much at all on the quality of blastocysts. And we know from clinical programs now in which single blastocysts are being replaced in the uterus that morphology scoring of blastocyst does correlate with pregnancy. So blastocysts are graded on the degree of expansion, the size and compactness of the inner cell mass, and the trophectoderm morphology.

And what we find is that if we grade these according to these criteria, is that these top grade ones have a pregnancy rate of 60 percent, the lower ones 33 percent, and low again we haven't been able to get pregnancies from. So what I'm trying to do is say blastocyst quality is important, and does predict pregnancy, and the information on this is currently lacking from the studies that we've seen. I think a starting point here is that women should have a fair chance at getting pregnant.

So some additional work that we've also been asked to do by the regulator in the UK with the Human Fertilization and Embryology Authority, they convened an

expert panel to look at these procedures, and they've made some recommendation about what work should be done, so in the next few slides I'm going to talk about those.

So the minimum set of experiments required, this was a document set out in 2011, is that we should see spindle transfer in human oocytes that are then fertilized. At that point we had seen spindle transfer and parthenogenetic activation, they wanted to see some that are fertilized. They wanted to see pronuclear transfer using normally fertilized human oocytes as opposed to abnormally. And they wanted to see pronuclear transfer in a nonhuman primate model at that stage.

So they recently met again last year and revisited the minimum set of experiments because there had been some progress in the field in the meantime. And so they acknowledged that the spindle transfer in human oocytes has now been performed, but would like to see some follow-up experiments to improve the efficiency, and they'd like for other cooperative experiments. They want to see pronuclear transfer using normally fertilized human oocytes, and that work is ongoing in Newcastle, and perhaps elsewhere, but Newcastle is the one I know about. And the recommendation for pronuclear transfer in a non-human primate model has now been deemed to be no longer critical. That was mainly on the basis of work that has emerged from

Dr. Mitalipov's lab indicating that when you come to the fine print there are differences between the rhesus monkey and the human, for example notably in the abnormal fertilization. So they just wondered about the value of doing the rhesus monkey experiments. And they also wanted to see experiments to characterize the segregation pattern of mitochondrial DNA variants and embryos in embryonic stem cells and in their differentiated derivatives. And they're happy to see this from embryos generated by PDD from affected women, or from embryos generated from these procedures.

So just to summarize my own view on the additional experiments that need to be done, I think significant insights into the likely efficacy of the new techniques can be gained by further in vitro experiments on blastocysts, and safety testing, including chromosomal constitution, gene expression, epigenetic analysis, and the segregation and fate of variant mtDNA. I think it would be important to perform those on blastocysts as well as on the ES cells. I don't mean to be negative about the ES cell work, I think it's very valuable and has been very informative, but I think we do need to look at blastocysts as well.

And in terms of these additional in vitro experiments, they will enable couples ultimately who might

benefit from the new techniques to make better informed decisions if they have more information. I believe it will also lead to further refinements of the procedures that are likely to improve their efficiency and increase the likelihood of success in any future clinical trial.

I was asked to speak about ideas for future clinical studies to test for safety and efficacy. I'm coming at this from a point of controlling variables where it's at all possible, control the controllable. So the way we would proceed with this in Newcastle if we were allowed to do this as clinical treatment would be to initially offer it to women with high levels of pathogenic mitochondrial DNA mutations, because at the moment we can offer PDT to women with lower levels, and we think until we get a feel for how these procedures go it's best to stick to what we know. And the risk benefit analysis for these people with high levels of mutation is more favorable. The initial investigations should be confined to young women with good ovarian reserve. This is important because female reproductive aging could have a majorly confounding effect on the interpretation of the data.

In terms of donors, something I didn't put in, which seemed too obvious, was that there should be no history of pathogenic mutations in the families. Younger women, less than 35 or younger if possible, preferably with

proven fertility. And as a starting point we would go for matching the mitochondrial DNA haplotype. I think this question is an open question, but it's a variable that can be controlled for at this point without too much difficulty. There should be follow-up during pregnancy, detailed analysis in the event of spontaneous abortion, and long-term follow up of any children born, as much as possible.

Just to go through the key points here, based on the low level of carryover, pronuclear transfer and spindle transfer I think we can accept will greatly reduce the risk of transmitting mitochondrial DNA disease. So that leaves us with a question of whether the procedures are safe or whether the mix of mitochondrial DNA is going to cause a problem. The evidence from animal studies and the in vitro data so far are encouraging, but I think further work is required to increase the probability of success of future clinical treatments and to better understand the risks.

In relation to the risks I think we can do a lot of in vitro studies to satisfy ourselves, but we can never go to clinical treatment and say this is completely safe. But what we can say with some certainty is that children that are born with high levels of pathogenetic mutations are likely to not be very well. These are debilitating diseases, and fatal diseases.

The last point is that future clinical studies should proceed with caution. That is to just control for as many variables as we can, and do it in cases where the benefits far outweigh the risks, and that would be high mutation loads.

So that's dealing with the mitochondrial DNA mutations. I just have a couple of slides at the end to deal with the idea of spindle transfer for age related fertility, because I know that has been mentioned, and the same arguments apply to actual transplantation of mitochondria into the egg. This is an egg going through meiosis, this is an immature GV stage oocyte, this is meiosis I, and this is meiosis II. So the oocytes have to lose have of its chromosomes before it can be fertilized. And in women, and especially older women, this process is highly error prone, and we know that greater than 70 percent of oocytes are affected by segregation errors in older women.

So this is normal segregation here, where we get a pair of homologues linked together at the time of recombination, they line up in the spindle, and then one homologue segregate to the oocyte and one to the polar body, that's a normal first meiotic division.

But we know from recent analysis, and indeed not so recent as well, during the whole genome replication of

polar bodies, that actually a very common problem in eggs of older women is that you get separation of the sister chromosomes that make up these homologues, such that you get this kind of configuration where you get gain of the single chromosome in the egg, or just one chromosome in the egg, which would lead to possibly monosomy. And then from earlier cytogenetic analysis we find that this separation of sisters and both being retained in the oocyte correlates with age.

The segregation errors and structural defects that give rise to aneuploidy have already occurred by the time the metaphase II oocyte is harvested from the oocyte, and it indeed is very difficult to envisage how a spindle transfer or indeed pronuclear transfer or indeed injection of mitochondria is going to correct those errors.

So this is my final slide, just to provide some information about how the process is going in the UK. In the last revision of the act governing human fertilization and embryology there was provision made for the introduction of these techniques specifically to prevent transmission of mitochondrial DNA disease, but that was subject to regulations being introduced by parliament to say that they were based on safety and efficacy. So the government indicated in June last year that they would draft regulations to amend the HFE Act. Those regulations

have not yet been published, but once they are they will go for public consultation, and then there will be a debate and votes in both houses of parliament, and then if it's passed detailed regulations will be agreed and adopted by the Human Fertilization Embryology Authority. That will enable them to offer a license to anybody who applies. Before they would grant a license they would review the evidence that's submitted to them.

So I'd just like to finish by acknowledging my collaborators, Doug Turnbull, who is a mitochondrial geneticist, and Alison Murdoch who runs the egg donation program, and this is the research team working on this. So thank you for your attention.

DR. SNYDER: We are open for question.

DR. KEEFE: The idea of focusing on real virulent types of mutations as a first pass makes sense. But even those that are 100 percent –- Nate Traph and Richard Scott published a paper last year where they used PGD, and they found up to tenfold variation in the level of heteroplasmy, even among oocytes, and even among the lymphocytes that they started with, suggesting that it's very hard to know who has homoplasmy. They too might have, even within their own leukocytes, a line that's less heteroplasmic.

So I'm wondering wouldn't an appropriate approach, imagine you're doing a study, wouldn't you want

to first try PGD to see if you could find some embryos that were – they found nine percent heteroplasmic, in others 90 percent of the same cohort, before your engineering things. And the second, related to that, is that if you do in fact focus on these women, these patients with 100 percent homoplasmy, does that have any impact on their labor and delivery? I know we have a number of MFMs here. Is that a concern that they're carrying, or would you also want to include a gestational surrogate if you're picking women that have some sort of subclinical mitochondrial DNA defect?

DR. HERBERT: I think in relation to the selection of patients for this, obviously it's going to be a decision made between gynecologists, obstetricians, and mitochondrial geneticists. But I imagine one of the criteria will be that the woman is reasonably well. In terms of the homoplasmic women possibly not being homoplasmic, we've had some patients with high mutation load and gotten normal embryos to replace. These are pictures that will emerge as we get more experience with PGD.

DR. MORAES: You mentioned that your colleagues said that women with mitochondria DNA mutations do not have reduced fertility. Is that right?

DR. HERBERT: That surprisingly seems to be the

situation.

DR. MORAES: How do you know that?

DR. HERBERT: Well, because they keep having affected babies. They don't not get pregnant, they have babies.

DR. MORAES: Are they really affected women, or just carriers?

DR. HERBERT: Well there is a famous case of the homoplasmic woman who has had seven children. It's surprising to me, and it just tells us that there is a great deal we don't understand about mitochondria and early development.

DR. CRIPE: A question about the grading that you do of the embryos and their quality. Does the pronuclear transfer affect the proportion of the grading, and does the grading still apply in those cases?

DR. HERBERT: I would like to be able to tell you more about this, but we are just about to submit it for publication and this is a public meeting. I think I can say from Shougot's(?) work, the blastocyst development itself is quite encouraging.

DR. SNYDER: Any other questions?

DR. GEARHART: If I understand you correctly the legislation does not say the work is going to go forward, the investigators must come to the HFEA with a plan, with

data, to get permission or approval. What is the word here?

DR. HERBERT: For a license. So you want a license to offer this treatment, and the evidence they would be looking for I imagine is going to be informed by the HFE, by the expert panel.

DR. SNYDER: Any other questions or comments?

DR. WENSTROM: I am just curious what's the informed consent process. I actually have taken care of women with mitochondrial disease, and ironically in my experience the most sick women would not want to be pregnant because they wouldn't tolerate it, and so the patients I've had with mitochondrial disease are relatively unaffected, but several of them have really done terrible during pregnancy.

We just had a patient who had multiple severe hypoglycemic episodes, found unconscious over and over again, and she did not have that problem when she was not pregnant. What kind of informed consent do you go through with these women to talk about the effects of the pregnancy itself?

DR. HERBERT: I think this is very much mitochondrial geneticists who have knowledge of these families and these conditions working with gynecologists and obstetricians to make those clinical decisions.

DR. SNYDER: I should mention that what informed

consent should look like is one of the questions that we're going to be addressing this afternoon or tomorrow morning. So that is quite important. If there are no other questions, thank you Dr. Herbert, and we can then move to the next stage of the proceedings, which would be open public hearing. And prior to the open public hearing I just need to read a statement into the record concerning financial disclosure and conflict of interest.

Both the Food and Drug Administration and the public believe in a transparent process for information gathering and decision making. To ensure such transparency at the open public hearing session of the advisory committee meeting, FDA believes that it is important to understand the context of an individual's presentation.

For this reason FDA encourages you, the open public hearing speaker, at the beginning of your written or oral statement, to advice the committee of any financial relationship that you may have with a sponsor, its product, and if known its direct competitors. For example, this financial information may include the sponsor's payment of your travel, lodging, or other expenses in connection with your attendance at the meeting.

Likewise, FDA encourages you at the beginning of your statement to advise the committee if you do not have any such financial relationships. If you choose not to

address this issue of financial relationships at the beginning of your statement it will not preclude you from speaking.

I'll call the names of the individuals who registered by the deadline to indicate that they would want to make public comment. Each person will be allotted four minutes. The first speaker is Marcie Denovsky.

Agenda Item: Open Public Hearing

MS. DONOVSKY: Good afternoon, I am Marcie Denovsky, I am the Executive Director of the Center for Genetics in Society. I don't have any financial conflicts of interest. I want to start by thanking you for the opportunity to speak today, and to note the fact that this public meeting is being held and the fact that this issue has already garnered a fair amount of media attention signals that it's a big deal, there is a lot at stake here.

In fact the briefing document for today's hearing acknowledges that ethical and social policy issues related to genetic modification of eggs and embryos have the potential to affect regulatory decisions, but the briefing document goes on to put these issues outside the scope of this meeting, which I think is troubling. I'm concerned that this means that very crucial societal and policy questions could be swept under the rug, and I really hope that is not the case. I hope that there will be proposals

for rigorous examination of these issues and for robust public discussion of these issues, and that this committee can make recommendations along those lines.

The elephant in the room of course is inheritable genetic modification. You may know that laws prohibiting intentional changes to the human germ line are actually on the books in more than 40 countries around the world. Many of these nations have highly developed biomedical and biotech sectors, and many of them have stronger protections for public health and for reproductive rights than we enjoy in the United State.

In fact, every country that has adopted any formal policy on human germ line modification has actually decided to prohibit it. The Council of Europe forbids these sorts of modifications in its convention on human rights and biomedicine, and UNESCO and the UN General Assembly have warned against this as well. Public opinion is overwhelmingly against it in opinion polls.

It is true as we heard that the United Kingdom is considering approving clinical trials of a mitochondrial manipulation technique, but if this happens it will be as you've heard after a vote of parliament, and it will be as a narrow exception carved out from the country's existing prohibition on inheritable genetic modification.

This bright policy line is there for a reason. As

many social scientists, policy experts, legal scholars, and others who have looked at this have concluded, if one kind of germ line change is permitted it does become more difficult to prevent modification of the nuclear genome, and to prevent that modification either for therapeutic purposes or even for purported enhancement purposes. The motivating concern in not going down that road is we want to avoid winding up in a world in which researchers or fertility clinics or insurance companies or governments or parents decide that they're going to try to engineer children with specific traits, and even possibly putting in motion a regime of high tech consumer eugenics.

Now, if you are inclined to dismiss such scenarios to say that's just a slippery slope, let's change the metaphor. Think about mission creep. We noticed that while these mitochondrial manipulation techniques were proposed for the prevention of transmission of serious mitochondrial disease, and I want to certainly acknowledge these diseases can be quite devastating, that was the initial idea, but now these techniques are being promoted in the pages of our nation's pre-eminent scientific journal as a way to address age related infertility. That's mission creep.

What we've heard in the briefing document, what we've heard this morning, these techniques are going to be

risky. And it may be that such high levels of risk would be arranged if we were talking about people with life threatening conditions, about treating them, and if there were no alternative treatments. That's usually how bioethicists think about this. But this isn't the case here.

These manipulations are not meant to treat people who are sick and suffering, we're not talking about people having mitochondrial mutations preventing them from developing illness, what we're talking about is radical experiments on future children and future generations, and I think Jeffrey Kahn at Johns Hopkins put it in a good way in Science last week when he said we're not treating humans, we're creating humans.

If the FDA were to approve clinical trials it might turn out that this would be the very first time any jurisdiction in the world had authorized intentional genetic modification of children and their descendants, and were the FDA to make such a decision it would be doing so without any input to speak of from the public or from elected officials. And I just want to say that I think a decision of such profound magnitude and consequence is not one that should be made behind the mostly unfortunately closed doors of this agency. Thank you.

DR. SNYDER: Thank you very much for your

comments. The next speaker is JD Hanson.

MR. HANSON: Good afternoon, I am JD Hansen from the International Center for Technology Assessment. Our organization has no financial conflicts related to this, and I have no personal financial conflicts related to this subject. I would note that one of the former staff of our organization began the work that led to the Supreme Court ruling that human genes are not patentable, so we have a long interest in human genetics.

Since this is the public part of the meeting I do think it is useful that we begin using public language. If the meeting were called to look at cloning technology in reproductive processes for the prevention of mitochondrial DNA, we'd probably have a lot more people here today.

I think that the FDA is the wrong agency to be looking at this. The very nature of the FDA's review process, looking at things essentially like drugs, where we look at them, where we have proposals that the FDA is legally required to keep secret, and I can't know that the people testifying today are all of the proposed trials that you have before you because your policies wouldn't allow you to disclose that.

The FDA when we ask questions about the ethics of animal cloning said we don't do ethics. There have been other reports that do ethics fairly well. I would point

back to the National Academy of Science Report looking at human embryonic stem cell research which was cochaired by an ethicist. I'm glad you're having the meeting, but I think that there are some other ways to look at the research too.

The National Academy of Science Report at that time actually had some instructions for this field. It specifically said that you shouldn't allow embryos that you have been working to engineer to develop beyond 14 days, that was the standard at the time, but we ought to debate whether we should keep those earlier standards. So we strongly believe that these techniques should not be allowed to move to clinical trials. We believe the PND in particular is essential embryo cloning, it was the technique that Dolly the sheep's creator used before they used somatic nuclear transfer. We would be concerned that this is sort of a gateway technology to using SCNT or other methods in human trials as well.

We also would urge that we look a lot more carefully at all of the animal research on cloning. This research demonstrates that there are major problems in the health of cloned animals. We heard a little discussion today on cattle, and probably the closest I come to a conflict of interest is I come from a family of Angus breeders. But one of my cousins who is a registrar for the

American Angus Breeders says that they're getting fewer clones registered because of some problems that they're seeing in the cloned animals, and they're not getting what they expect. Now, they're looking for meat and other issues, not health of the animals all the way. But I would urge the committee to look at the data on sheep, goats, pigs in particular. We're seeing there defects that pass down to the second generation, and I think that's relevant to look at in this.

The Chinese are doing large animal studies in cloning, I think you should look at some of that, with the carp and gold fish clones they found that defects related to mitochondrial DNA didn't really start showing up clearly until 500 animals. We have nothing close to 500 primates that we've looked at for this. Thank you.

DR. SNYDER: The next speaker is Stewart Newman. And again I'd like to remind the speakers to keep your comments to four minutes please.

MR. NEWMAN: So I am a developmental biologist. I'm a professor of cell biology and anatomy at New York Medical College, I'm used to looking at grant applications in developmental biology and reviewing papers, and I've been hearing a lot in the literature and here on mitochondrial manipulation, mitochondrial transfer, mitochondrial DNA transfer, and I didn't see one slide that

described any of that. In fact, the healthy mitochondria are in an egg which is receiving the nucleus or the spindle from another woman. The other woman is the intended mother, but that's just a legal issue. Biologically the egg is receiving a genome, or a haploid genome. So it seemed to me that there is a question of framing. If you frame it from the point of view of the intended mother it seems like sort of a small thing, she's just getting a couple of dozen mitochondrial genes. But she's not just getting a couple of mitochondrial genes, the woman's egg that has been enucleated and is receiving the spindle is getting 20,000 genes.

I have a couple of slides about problematic concepts in conventional description. There's the intended mother, the donor egg, the outcome, and the compatibility of the procedure. I'm not going to read this whole text, but you have the intended mother which is a legalism. There's the spindle donating mother, and she's only getting, as far as genes are concerned, mitochondrial genes.

But she's getting much more. She's getting an egg, the egg envelope, she's getting the egg cytoplasm which has all sorts of maternal factors, recent studies have shown that there is transgenerational inheritance that is via RNAs and proteins that are in the egg cytoplasm, the

oocyte cytoplasm. So this is not a trivial thing that even that woman is getting.

The woman who is the egg donor is getting a humongous compliment of genes from the other woman. She is in a way the mother also. In fact, we've heard about parthenogenesis today, the egg itself, even without fertilization, is capable of developing into a new individual, but a chromosomal spindle or a nucleus is not capable of developing by itself into a new individual. So from this woman's point of view this is a very weighty procedure.

But actually biologically speaking the most important point of view in framing this issue is the new individual that's being formed, and I don't think anybody can deny that this new individual has been the product or will be the product of a very massive procedure. Basically you take one oocyte and you remove the spindle from it, you take another oocyte you remove its nucleus, you put the spindle into the second oocyte, then you fertilize it. Framed from the point of view of the new individual this is a very large procedure.

And if this is approved, then in the future somebody comes back and says I'd like to put five new genes into a developing embryo, and they'd say well we only allowed mitochondria before. But no, you allowed 20,000

genes, not just the mitochondria. So what's a couple of genes? If you genetically engineer a tomato by adding one gene, then it's called a Genetically Modified Organism. Well, this new individual is a genetically modified human being, massively. Thank you.

DR. SNYDER: Thank you very much Dr. Newman.

MS. MS. AIRD: Good afternoon, my name is Anola Aird and I represent Mothers for a Human Future, a nonprofit initiative working to raise awareness, advocacy, and activism about biotechnology that have altered the human species. We do not have any financial conflict of interest.

I want to thank you for this opportunity to share our views. We very much appreciate the efforts to help people who have serious health challenges. But we're also deeply concerned about the future for children should mitochondrial manipulation procedures be approved. These procedures as it has been said are forms of human germ line modification that would engineer the makeup of future children.

These procedures are inherently unsafe. The FDA briefing document speaks to the many known and unknown risks that would be imposed on any resulting children and their dependents. It is simply too early to know what the long-term effects of these manipulations will be on

animals, let alone on human beings. These procedures would also involve an unprecedented level of experimentation on non-consenting human subjects. In this case, future generations.

You’re talking essentially about bringing into existence children whose genes would have been irreversibly changed using high risk experimental techniques when neither they nor their decedents whose genes would also have been irreversibly changed would have been able to give their consent. This raises such grave safety concerns that it alone should be sufficient reason to now allow human clinical trials. Allowing these procedures could also open the door to further germ line manipulations to the creation of genetically modified children, and a perversion of the relationship between parents and children that would treat children more like objects of design and manufactured products than human beings with feelings and emotions.

Allowing the creation of children with genetic material from three or more parents would open the door to the alteration of the human species and the creation of different biological Gattaca like classes of human beings and the dissolution of our common humanity. Because germ line modification could lead to biotechnological eugenics and the alteration of the human species over 40 countries have adopted policies prohibiting its use.

A decision to allow any use of germ line modification is a profound consequence to the entire human community, and it should not be made unilaterally by this committee, by the FDA, or by any one country. Mitochondrial manipulation technologies raise issues of profound significance to all human beings, where there is relatively little public awareness or debate about them. There is a pressing need to maintain the existing worldwide moratorium on the use of germ line modification to allow for broad based international public education, conversation, and decision making on the appropriate regulatory framework to promote responsible uses of these technologies.

With all due respect this issue is much too important to be decided within the confines of this room among these people. Hopefully this committee and the FDA will be leaders in the effort to fully educate and engage the public in the decision making on this crucial issue. I urge this committee and the FDA to consider the moral, the ethical, the legal, and the societal and international implementations of its actions, and to say no emphatically to human clinical trials of mitochondrial manipulation technologies. It would be irresponsible at this juncture to do otherwise.

DR. SNYDER: Thank you very much for your comments. The next speaker is David Prentice.

DR. PRENTICE: Thank you. Good afternoon, I'm Dr. David Prentice, I'm Senior Fellow for Life Sciences with the Family Research Council. Prior to that I served two decades as Professor of Life Sciences at Indiana State University and Adjunct Professor of Medical and Molecular Genetics at IU Med School. I have no financial conflicts of interest.

It's my understanding that you've received the written comments, so I just want to give you some brief oral points at this time. FRC opposes approval, as well as funding, or any guidance issued for these techniques. Number one, the proposed techniques are all non-therapeutic. In point of fact what you're discussing is creation of new human individuals and not treatment or therapies for anyone who is currently affected by these particular mitochondrial mutations.

The proposed techniques treat human life instrumentally, including destruction of human embryos for experimental purposes. All the techniques discussed manipulate human life and treat new individuals as experiments. Pronuclear transfer technique relies on destruction of two embryos, one completely healthy, to manufacture a third recombined embryo.

Number three, the proposed techniques all treat women instrumentally. All the techniques rely on a

significant number of donor eggs with healthy mitochondria to accomplish their ends, and the typical procedures used for donations of eggs exposes these young women to significant health risk from ovarian hyper stimulation syndrome, including uncertain long-term health risk.

Number four, the proposed techniques all promote germ line genetic modification. All of these techniques by definition alter the germ line of any human embryo created by the techniques, and these alterations will be passed to the offspring of the modified individuals with unknown consequences.

Number five, these proposed techniques all foster human cloning. All the techniques foster the skills needed for human cloning via nuclear and egg manipulation. In point of fact, pronuclear transfer as well as a related technique which could be used, embryo-cell nuclear transfer, technically are cloning of a human embryo for the purpose of reproductive cloning, or cloning for live birth.

Number six, the proposed techniques are not safe. There are significant concerns about the safety of the mitochondrial transfer and the oocyte modification. Ooplasm transfer is itself associated in mice with decreased viability of offspring as well as other subtle effects on growth and development. And there have been recent publications documenting this.

Though it's not the purview of the committee, it was mentioned previously in some of the talks, I'd like to point out that federal funding of these proposed techniques is illegal. Any federal funding of the proposed techniques violates the legal prohibition on funding research in which embryos are created, harmed, or destroyed, a provision known as Dickey-Wicker amendment, which has been passed by Congress and become law since 1996.

One other point I want to raise related to my first point about this being non-therapeutic is it seems you have focused very much on these particular creation techniques without looking at any alternatives or considering alternatives which might actually treat affected individuals. And I've listed some of those in the written.

For example, recent evidence for transfer of mitochondria from donor bone marrow adult stem cells to other tissues, evidence for alteration of mutant mitochondria using talons, as well as CRISPR technique. And in fact there was a paper published in science 20 December when use of the antibiotic rapamycin alleviates mitochondrial diseases in a mouse model of Leigh's syndrome. We would urge you not to approve, nor even to issue guidance on these techniques. Thank you for your time.

DR. SNYDER: Thank you Dr. Prentice. The next speaker is Sheldon Krimsky.

MR. KRIMSKY: Thank you for the opportunity to speak. I'm Sheldon Krimsky, I'm on a board of the Council for Responsible Genetics, I'm also a professor at Tufts University in both arts and sciences and the medical school. I also served on the Recombinant DNA Advisory Committee where we had grappled with many of these issues of human gene therapy, and I was an advisor to the presidential commission report Splicing Life, which dealt with human genetic engineering.

The first reported human pregnancy following cytoplasm transfer, and I'm going to be focusing exclusively on cytoplasm transfer, took place in 1997. Like many developments in assisted reproduction it was designed to help pregnancy, and that of course is a noble objective.

I offer three questions that should be answered before the procedure moves forward to gain FDA approval. One, is cytoplasm transfer safe and effective for the offspring? That is such a critical question. Secondly, if the procedure is found to be generally safe with some risks, do prospective parents have the authority by themselves to undertake the procedure balancing risks and benefits without additional oversight? And third, are the potential benefits of cytoplasm transfer from improving

fertility or preventing the transfer of mitochondrial disease unique and sufficient to open the door to germ line gene modification, which has been discussed by several of the people before me.

I'm just going to address question number one. Most scientists who specialize in the biology of reproduction and who have written about cytoplasmic transfer have a clear message. Cytoplasmic transfer appears to be consistently associated with mitochondrial heteroplasmy. That's point number one.

Point number two, heteroplasmy, or babies born with two distinct female mitochondrial genomes, is a risk which must be understood before cytoplasmic transfer is considered for clinical practice. While an estimated 30 babies have been born using this technique there has never been a systematic follow-up of this cohort that examines the rate and degree of heteroplasmy in the newborn, and in cases where it exists, on its effect of the developmental health of the child.

So the few published animal studies report clear and present danger. I'm just going to summarize my review of the literature. Heteroplasmy created by a mixture of cytoplasm from different strains of mice resulted in physiological impairment, including disproportionate weight gain and cardiovascular system changes. Cytoplasm transfer

used in cattle produces heteroplasmic offspring. Some children born through cytoplasmic transfer have been identified as heteroplasmic.

There is crosstalk between mitochondrial DNA and nuclear DNA. It is not known, but suspected, that nuclear DNA crosstalk between two mitochondrial genomes will affect the development of the offspring. This has to be investigated thoroughly. The paternal genome may be especially susceptible to epigenetical alterations by foreign ooplasm. Mixing of two different mouse mitochondrial DNA within the same female germ line can lead to offspring with neuro-psychiatric defects, an important study. While offering the prospects of treatment to some infertile couples, cytoplasm transfer is quite capable of generating unexpected abnormalities.

Probably the most current and comprehensive review article of mitochondrial DNA and heteroplasmy is found in Cold Spring Harbor Perspectives in Biology, where the authors say all appropriate pre-clinical tests must be performed in an effort to reduce the risks for an adverse outcome.

Of course, everyone on this committee probably knows the Jesse Geisinger case, where animal studies were not done significantly and sufficiently to prevent the death of a 19 year old from human gene therapy. Many

questions need to be answered before cytoplasmic transfer should be considered safe and effective to the offspring. Until these questions are answered, first by systematic animal studies, I can find no consensus within the scientific community to proceed. That's my reading of the scientific literature.

And then there's just one thought experiment I'll ask you to think about. supposing that there are risks and a child is born with cytoplasmic transfer and heteroplasmic condition, and has defects from this process, and the child needs to be cared for quite considerably, and you ask the child how important is it for you to have had your mother's nuclear DNA or to be a healthy child, I suspect the child would say I wish I were born as a healthy child, the nuclear DNA is not that important. Thank you.

DR. SNYDER: Thank you very much Dr. Krimsky. The next speaker is Juliet Isenberg. Is Miss Isenberg here? We'll move on to the last speaker, Joy Riley.

DR. RILEY: Good afternoon, I am Dr. D Joy Riley, the Executive Director of the Tennessee Center for Bioethics and Culture, an educational not for profit organization headquarter in Nashville Tennessee dedicated to promoting human dignity in the face of challenges to what it means to be human, and to informing and equipping people to face the vital bio-ethics issues of our day. I

have no conflicts of interest to report. I'm a physician by trying and hold a graduate degree in bioethics as well. I appreciate the opportunity to speak with you.

It is remarkable, as other people have noted, that more than 40 nations have prohibited germ line modification. In the wake of this we are contemplating stepping over that bright line. It is imperative that we reflect upon the words of philosopher George Santayana, who presciently said those who cannot remember the past are condemned to repeat it. This applies in several ways.

First of all, consider that this change is being evaluated by the Cellular, Tissue, and Gene Therapies Advisory Committee of the FDA. While this is your purview, it is not merely cells, tissues, or genes that are being affected by this decision, it is human beings, human beings at their earlier stages, who are the subjects of this research.

These are your and mine children, grandchildren, nieces, nephews, and cousins. Their petri dish appearance should not confuse us, they are very much human. We as a nation do not have a good track record recognizing who qualifies as human beings. In fact in our early years we counted some adult men and women of African descent as less than whole persons, specifically as three fifths persons. The fourteenth constitutional amendment was a remedy. Those

who cannot remember the past are condemned to repeat it.

Secondly, consider codes of international standing. The Declaration of Geneva proclaimed for physicians worldwide the health of my patient will be my first consideration. The Declaration of Helsinki stated in medical research involving human subjects the wellbeing of the individual research subject must take precedence over all other interests.

Both these declarations were lessons learned from World War 2 in reaction to human experimentation atrocities uncovered by the Nuremberg Trials, and Unit 731, a prison experimentation camp conducted by the Imperial Japanese Army. In the Council of Europe’s Convention on Biomedicine and Human Rights, intervention on the human genome is countenanced, in quotes, only if its aim is not to introduce any modification in the genome of any descendants, end quote. Those who cannot remember the past are condemned to repeat it.

Finally, a major requirement of research on human subjects is that a fully informed consent by the human being whose life, health, and posterity will be impacted by the proposed human experiment. How does such informed consent work in this research proposed? The men and women from whom the sperm and eggs are procured are conceivably persons with capacity and able to consent to the

procurement of their gametes.

Egg harvesting is not without risk, but we have to understand that the persons who bear the weight of this experimental risk are not the adults consenting to procurement. The humans who bear the weight of this experimental risk are not even being treated, because this is not therapy for ones undergoing experimentation. Their very formation is the experiment under consideration. They are not able to give consent, neither are all their descendants who come after them, yet the proposal is to experiment on all of them by virtue of altering their germ line for all time.

I submit to you that proxy consent is wholly inadequate. The FDA jettisoned the Helsinki Declaration several years ago and substituted rules of elements of informed consent. Each subject must be informed that this is research, they must be told the explanation of the purpose of the research, the expected duration of the subject’s participation, as well as given a description of any procedures which are experimental.

So let’s read that consent form that you will present to the next generation of a three parent embryo. This research project began before you were conceived. It has fashioned you as we see fit. It will last all of your life. It will affect all of your children, and their

children after them. Have you ever agreed to participate? Sign here. Thank you.

Agenda Item: Committee Discussion

DR. SNYDER: Thank you very much. The committee thanks the members of the public for their thoughtful comments, and we'll certainly take your thoughts into consideration in our discussions. Because we're somewhat ahead of schedule, rather than taking a break now I think maybe what we would do is begin discussion of question number one, go for about 45 minutes, and take a break then, and then in the last part of the afternoon tackle question number two. Is that agreeable to everybody?

For those who are not used to how the committee deals with questions, we'll be doing it the following way. I'll read the question, but you'll also see it projected onto the screen. And then the discussion will be kicked off by three lead discussants that will simply give some initial thoughts on the various parts of the question, and then it will be opened up to the entire committee. I encourage everybody to please make some kind of comment, and at the end I'll try to summarize what our thinking is.

And inevitably I think the discussion in question number one will feed into question number two, and then into three and four tomorrow. So we'll constantly be revising our thoughts. But I do encourage everybody to

please participate so that we get a really full scent of the committee and we can offer some of our advice and some of our thoughts to the FDA.

Discussion topic number one: The goal of mitochondrial manipulation technologies is the prevention of transmission of mitochondrial disease from an affected woman to her children, or the treatment of infertility. Prior to human clinical investigations animal and in vitro studies provide the primary data upon which safety assessment is made.

In the context of the available animal models or other experimental systems for mitochondrial manipulation technologies, please consider the specific objectives of studies that would be necessary to support the safety and the prospect of benefit of mitochondrial manipulations technology prior to first in human clinical trials.

Please discuss the ability of available animal models and/or in vitro methods to address the following: A, the possibility of inadvertent damage to the manipulated oocyte or embryo. B, long-term risks associated with the carryover of abnormal mitochondrial DNA and heteroplasmy in the children. And C, the potential for abnormal embryo/fetal growth resulting in children with significant defects.

To help kick us off and lead the discussion and

share some of their thoughts on these topics, Linda Dahlgren, Theresa Woodruff, and Carlos Moraes will give some of their initial thoughts and hopefully stimulate some discussion. Linda?

DR. DAHLGREN: Thank you. So I am not sure that I have the best background for making comments on this subject, so I'm just going to speak generally. But based on what I was provided to read and what was presented today, I don't see that we have abundance of evidence in animal models or even really in the in vitro information that's presented. There is some compelling information, but in my mind it's certainly not substantial as far as convincing me that we're at a place where we're ready to move forward with human clinical trials.

DR. WITTEN: Can I just clarify the question? I think we are not asking if the information is good enough to go forward, we're asking to explain the ability of available animal models or in vitro methods to generate data for us to go forward. So what we'd want it actually discussion of the ability of these methods to address the various risks.

DR. DAHLGREN: I am happy to discuss that. Certainly the in vitro studies are able to investigate the safety to a limited extent. So how did the blastocyst look? And in the same extent then, the next step being in animal

models, I think mice are a good model for certain things, and to a limited extent. But mice are definitely very different from humans or even other larger mammals, so I think there are some significant limitations there. Other information presented today was on primates, which I think certainly are closer to humans, there do seem to be limitations there as well. I know that's not a very in-depth set of comments, but that's the best that I have to offer.

DR. SNYDER: Let me reiterate what Celia said. It's fine to indicate whether you think the data are in place right now for us to move forward, but more to the point is what would you want to see before you would feel comfortable. If you don't presently feel comfortable, what would make you feel comfortable to address these three particular aspects: whether there is or is not damage to the oocyte and embryo, whether there is or is not carryover of mutant mitochondrial DNA, and whether there is or is not risk of long persistent abnormalities in the offspring of the children. Theresa Woodruff?

DR. WOODRUFF: Thank you very much. I think this has been a really provocative conversation, and I think I too have thoughts that are a little bit scattered, so I'll try to coalesce them as well into something cogent. I think the first thing that strikes me is 2002-2014 is a lot of

time, and so I applaud the FDA for bringing this topic back into public discussion, because in the absence of this type of discussion there is no vehicle for this kind of deliberation, and particularly for discussion about what additional advances in basic science could help address the clinical problem as it's outlined here for us.

The first topic is really about animal models, and today we had extraordinarily cogent speakers talk to us about what their labs and the overall community has achieved on a number of different systems. I'm convinced that the bovine is a good proxy for human, and there is a lot of evidence in mono-ovulatory species that the bovine oocyte and its folliculogenesis represents many of the hallmarks of development that makes it probably the most tractable model for us to use. It does have downsides, as do any animal models, but its epigenetics, its proxy to human folliculogenesis, it's a mono ovulatory species, the way the spindle moves in the bovine parallels what happens in the human, unlike for example the mouse.

But of course the mouse has extraordinary power for us and should be continued just as the bovine because of its fast generation time and because of the neurocognitive assessment tools that are available in the mouse world that would allow some analysis of the offspring in sophisticated ways that we couldn't do for example in

the bovine.

The rhesus studies are the closest we've had to being able to model human disease. Of course, the NIH has restricted the use of the most comparable model to human, which is the chimp, and so the rhesus is a more divergent model, but still represents our best case scenario for unraveling the biology of oocyte maturation, the conversion of the oocyte to an egg through terminal meiosis, and then the transfer from a meiotic cell into now a mitotic embryo.

And so those rhesus studies should continue, now linked to the neuro-cognitive outcomes as well as metabolic challenges. That's going to rely on, as we heard, a combination of FDA guidance but as well NIH funding. And these studies are long-term and will require some significant investment to ensure that these animals can continue to be evaluated in a way that's going to be informative to the public on the relative safety of this kind of intervention.

In addition, the human studies really require that there be volunteers that permit induction in order to provide research based gametic material oocytes for these kind of research studies, and we heard about studies where seven volunteers were stimulated in order to acquire that particular material. We also heard that patients who would benefit from the research were solicited for these research

studies but were not interested in research, but rather wanted to wait for a clinical trial.

So one of the things that I think as we look at the different models as we move towards clinical trials, all of these being on the research side of the equation, is to help the patient population understand what the value is of research to their long-term perspective in order for us to learn something about the biology of their disease in order to better enable us as a field to move forward a clinical trial should the safety be well described by these models.

The question asks us to consider both mitochondrial disease as well as infertility, age, obesity, mitochondria criteria. These all represent really the cutting edge of basic science, and I think one of the keys for all of us is to recognize that there is a lot of room for us to understand something new about basic cell biology, how the mitochondria divide, how they're partitioned, how they're anchored within different spaces within somatic cells as well as germ cells represents the real next generation opportunity within this field, and should be therefore encouraged.

I do think that there is value from the UK model, which is really fundamentally a center of excellence. It makes sure that there are technologies that are placed

within institutions like Newcastle and I guess the seven or eight others where there is technical expertise. And this speaks to the other points that are listed below, the possibility for example of inadvertent damage to the manipulated oocyte or embryo. Certainly the word inadvertent is true. One would not want to intentionally damage that egg or embryo, and there certainly is the possibility for inadvertent damage based on existing ICSI or IVF technologies. And a way that the UK has decided to work towards the very best techniques is to have investments within centers of excellence that have the very best technologies developed.

And I think as we heard from the Oregon group one of the reasons for their success is because of the long-term technical experience that that group has that allows them to basically manipulate the oocytes in such a way that there isn't that inadvertent damage that is listed under your bullet A. The issue of long-term risks that are associated with transference of abnormal mitochondrial DNA, I think the purpose here is to mitigate or reduce the risk, and if one is going down this trial one should continue to do PGD I order to select the embryos that would have the highest likelihood of giving rise to a health offspring.

The potential for abnormal fetal growth in the offspring with significant defects, I didn't understand the

conjunction at the end, with those significant defects, so I'm not sure that I could speak to that very competently. I will put just for the discussion that we've heard largely about animal model systems.

There are in addition, as was mentioned by Dr. Sirard, there are in vitro follicle growth systems that are being developed. An L-carnitine study was used to try and enhance mitochondrial function within the context of a developing oocyte, and that gave rise to high quality blastocyst. So there may be potential for intervention at a point that hasn't been discussed by any of the discussants to this point, which is rather than work with the mature gamete after hormonal induction, to look back at the earliest stages of oocyte biology and to manipulate perhaps the mitochondrial function pharmacologically to permit that egg to develop a mitochondrial complement that may then support ultimately healthy offspring.

This certainly I think in the context of the second half of your discussion point, which is about the infertility, and particularly the age related idiopathic infertility where aneuploidy represents about 30 or so percent of many of the cases. That movement of the chromosome may be subordinate to a mitochondria that's unable to provide the energy to allow the spindle to be formed appropriately.

So certainly if we could interdict to support the mitochondrial health in those aging oocytes pharmacologically it would eliminate the need for any additional manipulation of that egg. And so an in vitro follicle growth system, which has been used in mouse, in cow, in cats, in dogs, in sheep and goats and in human, may provide a new model system for being able to move forward here.

I would note that using gametes that are derived from IPS sources may also provide new ways to understand mitochondrial specification within the germ line versus the somatic cells, and I think that's a really active area of research that should be encouraged, even as we're trying to get to that definitive high quality gamete we can be looking at the mitochondria as part of the genetic material that I think is often not evaluated in this kind of research.

Finally, I do think that there is real room for global research discussion, and perhaps global consortia that are going to look at some of these issues, because I think that there is enormous interest in being able to understand some of the fundamentals of the science here, and no single lab or single center will be able to do something like this on their own. So in order to ensure that we can best avert damage and provide low risk to

offspring and to ensure safety and efficacy, I think some kind of global perspective would be invaluable in this discussion. Thank you very much for the opportunity to discuss topic one.

DR. SNYDER: Thank you very much, and thank you for being so specific. I would like to encourage that all speakers, whether you're a discussant or just commenting, to be as specific as possible in order to provide guidance for the FDA. Basically this question is dealing with pre-clinical studies that will be informative. The second question will deal with being very specific about the risks, and then tomorrow we'll actually have the opportunity to design what we think a first in human clinical study should look like to give us data, and also to talk about manufacturing. This is our opportunity to be very specific about what metrics you would want to see for pre-clinical studies that would make you feel comfortable with advancing. With that, Carlos?

DR. MORAES: So from everything I heard today I think the main roadblock or the main concern is a technique of manipulating the chromosomes and not causing damage, and make sure you don't have aneuploid, and you have the right number of chromosomes. One question for the experts in this area is once you make an embryo and the embryo looks good, in the models that have been used around, how often the

individual, the mouse or the cow that's born will have a defect. So once you a good embryo are you going to have a good animal coming out of it? So that's an important question, and one that to me was the most concerning one.

In terms of item B that I know more about, I can talk a little bit more about it. So one concern that has been in the write ups and some paper is the compatibility of nuclear DNA and mitochondrial DNA. Recently I think I read a news piece that said about 80 percent of the published articles are wrong. I don't know if it's that high, but we cannot rely completely on published papers that may show some problems with compatibility.

But in nature, as Dr. Egli explained, if you have an English person that goes to Australia in the 1700s and has a girl with an aboriginal, and this girl goes back to Europe and marries another English person and has another girl, and this goes on and goes on for a while, and you have someone who has the mitochondria from an aboriginal Australian and the nuclear DNA from a European, and there is absolutely no evidence that there is a problem of compatibility of mitochondrial DNA and nuclear DNA. So I think this is good evidence to not be too concerned about the compatibility issue.

Now, there is the second problem that is heteroplasmy. And this also has been discussed a lot, and

there are a few papers saying that heteroplasmy is a problem. Now, I don't know if these papers are right or wrong, but if we look at other evidence that's a little less controversial, we see that heteroplasmy does not seem to be a big problem.

For example, in families that are carriers of mitochondria disease, you can have individuals that are heteroplasmic with a bad mitochondrial DNA that has a mutation that's pathogenic, but these people are completely normal because the levels of heteroplasmy are up to 70 percent in many families. And there are many cases like that, and also we already know and was discussed here that we are all heteroplasmic. I mean the evidence is really not there that heteroplasmy is a big problem, unless you have a pathogenic mutation and these levels are really high.

So in models that are available I think there are some mouse models, up till now there are not some great models of mitochondria DNA heteroplasmy that are pathogenic. There is one in Japan that has a deletion of mitochondrial DNA that is heteroplasmic. Unfortunately they do not share this mouse with the rest of the world, but they've done quite a bit of work. And again it shows that only when you have the mutant at very high levels you have a problem, so heteroplasmic per se is not a problem.

There are a few other mouse models that have been

generated and are going to be more available, but they also support the idea that heteroplasmy is bad only if the bad mutation is present at the high levels. And since we're talking about models we have hundreds of papers in culture systems showing the same, that heteroplasmy per se is not a problem unless the mutant is very high. And this is tested with at least 30 different mutations or more.

One question that I asked before, and we really have to talk about later, because maybe someone here has done these experiments, is if you introduce somatic mitochondrial DNA into an oocyte, if that improves fertility or it's bad for the oocyte. I mean this is just a topic of discussion that I thought to put in there.

In terms of the potential for abnormal embryo-fetal growth resulting in children with significant defects, again from what I've seen the main problem would be with chromosomal abnormality. So if we can get these techniques and we have enough models to test and see if you can make good embryos, if these embryos are going to be good, I will be comfortable with that.

We're not discussing ethical issues here, but for some families it's important to have offspring that have their genetic makeup. I'm sure that if a family has a child that's healthy because of a procedure like this, the child, once he grows up, will be happy that he's alive and doesn't

have that part of the DNA that was sick. So I think that we should look at this other side, if the evidence looks good, now once you have a good embryo you might have a good offspring, that's something to consider.

Just a last thing is the discussions in the United Kingdom. They had quite a bit of public discussion, and once all the facts were explained most of the population was not against that. Again, we're not discussing this, but having the basic facts explained is important. For example, the difference between cloning and this, they're very different. And I think I'll let the rest of the committee discuss now.

DR. SNYDER: Great, we will now open the floor up to everybody to make comments. Dr. Lee is going to kick us off, and then we'll go around the table.

DR. LEE: This morning there are many interesting talks. Apparently this mitochondrial manipulation technique seems to be important technology, but I haven't heard about any of the animal model, what is the sample size. How do we go to human child before we know what the sample sizes of these animal studies that were done?

DR. SNYDER: Maybe the most efficient way would be, since Jane raised her hand we can start there, and then what we can do is go around the table. If you feel that you've said everything you wanted to say you can just pass,

but why don’t we start with Jane?

DR. LEBKOWSKI: Okay. I want to comment again on animal models, not necessarily knowing which are the absolute best animal models, but the kinds of things I would like to see shown in animal models. I think as was said before one of the most important things is to look at what do the manipulations themselves do to the embryos. In this particular case, using the examples of nuclear transfer. It’s not the same thing, but using examples of nuclear transfer.

And when it was done in the 1990s to look at cloning and formation of sheep and pigs in particular, what we found was that there was an awful lot of abnormal fetuses, and the sample size didn’t have to be that big to be able to detect them and see those particular abnormalities in the fetuses and the abortuses that happened. So I think that in some model, probably the bovine that was suggested, we’d want to see that the manipulations themselves lead to the viable creation of embryos that can make it to term, that are normal.

I think the second thing we want to look at, again as was said probably in a mouse model, is looking at the various manipulations using mitochondrial DNA or mitochondria, can we actually correct a defect. And I think that that’s important to look at, and what the fidelity and

the penetrance of correcting that defect is.

I think the last thing that was mentioned a little bit prior was as an exploratory work what can IPS cells be doing to help us understand a little bit more the biology of the mitochondria in these particular defects, and can we examine in this particular case the effects of heteroplasmy, at least in somatic cells, and maybe even oocytes that are derived from IPS cells. Thank you.

DR. DIEKEMA: Again I am far from an expert on the science here, but I guess I have a couple of comments that I will frame as somebody that chairs an institutional review board and would be potentially reviewing these studies if somebody proposed a human study. In that regard I think I would want to see the kinds of studies in animals that approximated as closely as possible the kind of study that was being proposed in humans, which would include relatively long-term follow-up. So that’s comment number one.

Comment number two is I would also be looking for a sufficient number in the animal studies. Not one or two, I’d want enough to convince me they would most likely have caught relatively rare problems. And finally I see these studies in humans as more problematic for infertility than mitochondrial disease, and so would have difficulty I think feeling comfortable approving a human study in infertility

before those studies had been done for mitochondrial disease.

DR. KEEFE: I would like to see more animal studies. There are a lot of differences between the technologies we heard about today and the ones that lead to Dolly concerns, the stage of the differentiation of the chromosomes, as Dr. Egli said, the spindle transfer, they're concordant with the nucleated eggs. So the cytoplasm and the spindle are actually concordant in terms of their development. But we need to know that. It would be nice to see similar studies with spindle transfer in cattle and sheep. It makes sense.

And then there must be some way to get the less expensive mouse model to exhibit, as Dr. Moraes said there are some early studies of mitochondrial DNA mutations in mice, but there is a mouse strain for everything. I think if we could get those and use those to really drive the science it would be a big breakthrough.

DR. WENSTROM: I am clinical biologist, so I spend a lot of time doing pre-natal diagnosis, which involves identifying a genetic defect and then trying to predict the phenotype. So when I think about what we need to do here I think we need to really develop some kind of testing techniques to follow not just embryos but these animals through their adult lives, because when you think about

what happens in actual mitochondrial disease there are lots of patients that don't develop symptoms until adulthood, because presumably the number of mitochondrial mutations is increasing gradually in certain tissues and not others. Why does someone have 20 percent abnormal mitochondrial DNA in their lymphocytes, but 70 percent in their skeletal muscle, and no abnormalities in their brain, or the reverse?

I guess it makes me nervous when we talk about how looking at the blastocyst and if it looks health and if we decide it's not aneuploid then it's good to go, because there are so many aspects of mitochondrial disease that we don't understand, things that change over development that are tissue specific and response to environmental stimuli probably.

And so I think we really in an animal model need to figure out how to test all these tissues to really determine the safety of this technique over the lifetime of the animal. And the problem is there's really no good animal model that's exactly like a human, that's why so many teratogens were released before we realized a mouse is not like a human. And so that raises a huge issue.

We already have the example of imprinting defects resulting from the IVF techniques we use now. Nobody predicted you could get Beckwith-Biedemann syndrome from IVF, because that didn't happen in an animal model, and I

think we just have to be very careful that we develop techniques to really test these animals throughout their lifespan, all their tissues, in the very best animal possible, which I think has to be a primate. That's a very expensive and labor intensive process.

DR. STEINBOCK: I am also not a scientist, and so I'm not going to relate to that question. But I would like to follow up on what Kathryn just said, because this is a problem in every area of science, nothing is a completely good model. Sometimes when stuff has been tried in animals and found to be safe it wasn't safe in humans, as we all know from the Thalidomide scandal. So what do we do? We're left with a kind of a difficulty. And I think then the issue becomes how safe is safe enough and how much do we allow people to decide for themselves which risks are they willing to take.

And so I was thinking of a thought experiment, which would sort of be the flipside of the one that Dr. Krimsky suggested in the comments to the panel. He talked about the child who was born after mitochondrial manipulation technology who had a terrible disease, who said how important was it to you to have your mother's nuclear DNA or wouldn't you rather be healthy. But if we flip it on the other side we can imagine a woman who has mitochondrial disease but doesn't have any affect, doesn't

have any symptoms, and she wants to have a child, and she wants to know what her risk is. Well, what is her risk? We heard different things today. One in 24, we have a no clue, one in ten. So we don't know.

So if she were to take her chances and have a child, she might have a child that was disease free or she might have a child that has a disease. Was she wrong to do that? So if you think that people have the right to make that decision for themselves, it seem peculiar to say that a woman who says I'd like to avoid passing on this mitochondrial disease, I'd like to have a chance of having a healthy child, is doing something that is absolutely wrong. That's the only comment I wanted to make.

DR. SNYDER: Did you want to add anything more, Carlos?

DR. MORAES: I share your concern of the long-term consequences, but that will be more of a concern if the embryo has like 20 percent mutant mitochondrial DNA. If it has less than one percent it's very unlikely, from every system we study, that's going to accumulate to a level in the muscle or brain that's pathogenic. So if we can get to that low level of mutant I think it is unlikely that will become a problem.

DR. WENSTROM: I think in clinical practice, in our syllabus we were given a lot of information about

specific mitochondrial disease, with names, where we know exactly what all the features are going to be. But in clinical practice there are lots of people who have some features of these diseases, not others. They develop over the course of their lifetime. Maybe their first symptom isn't until they're 30, which suggests to me there's something fluid about mitochondrial mutations.

I think it's likely that you could have two percent mutations in your skeletal muscle at birth, and by the time you're 40 you have enough that you have symptoms, and that probably accounts for people that don't fit these specific disease categories. I think that's the most likely explanation for what we see in clinical practice. If that's the case, then how are we going to assure that this technique doesn't cause that, unless we follow the animal through their whole lifetime and continually test all their tissues?

DR. MORAES: We do not have any example of that, something with that low level that will increase to a pathogenetic level.

DR. WENSTROM: In patients you can test different tissues and they'll have very low levels in one tissue and very high levels in another. How does that happen? We don't know how it happens in humans, but we need to figure out how to monitor that in an experimental animal.

DR. MORAES: We believe that's during embryogenesis. Some tissues, the mutant will segregate at higher levels, and others will segregate at lower levels. So you may end up in blood with two percent and in muscle with 50 percent.

DR. WENSTROM: If we are going to do this manipulation we have to make sure that we identify that happening. If whatever we do causes something to rise 50 percent we have to figure that out before we adopt it for human.

DR. MORAES: But if you start in the embryo with less than one percent it's very unlikely that any tissue will accumulate 70 percent, from the examples we've seen. Humans not so much. But we don't have an example of going from such low levels in the embryo to becoming pathogenic levels.

DR. WENSTROM: Here is the problem: you can't test every cell in an embryo, because that would be the end of the embryo, right? You can only test a couple of cells, so you don't really know what the level is in the other cells. That's one of the theories about why you end up with such different levels in humans, is that some cells have virtually no mutations, others have high, and then mutation level may increase over the course of a lifespan. I'm just saying I think if we're going to have a mouse model that's

something we're going to have to really watch for.

DR. MORAES: I think this is super important, it's key for this discussion. And if anybody has anything else to say about this I think that might be a good moment.

DR. SNYDER: Did you want to add anything else to your earlier comments? Theresa, did you want to add anything more?

DR. WOODRUFF: One of the other things to evaluate for the animal or in vitro models is the aspect of cryo-preservation. So when the spindle is removed, and if it's vitrified for later transfer, which I think is the way Crysta was born, the few mitochondria that go along for the ride, the question is whether or not they are functional once they are thawed after that procedure. That may also help evaluate what the likelihood of transferring of those mitochondria may be. They may not functionally be able to be recovered after cryo-thaw.

MS. MS. REEDER: Thank you very much. I first want to actually apologize ahead of time for any of my comments just really out of ignorance. I am not a doctor, I am not a researcher, and I am not a scientist, and I have the utmost respect for all of the people around this table and the minds that are in this room, and the presentations that I listen to. I would just like to make some comments I think primarily addressing topic two, but in a general way,

in the spirit of just being a mitochondrial patient and somebody that lives with mitochondrial disease. So that's the place that I'm coming from, and some of my comments and some of the things that came up, I think I have more questions than any answers after listening to all the information.

My first thought is how can we prevent when we can hardly diagnose. And mitochondrial diseases are not rare, they're just misdiagnosed, and they're very hard to get diagnosed. I was diagnosed 14 years ago, and it took me 16 years in and out of doctors' offices saying something was wrong to actually get a proper diagnosis. My first symptom appeared when I was 18. I had a very droopy eyelid, and they just fixed it, nobody asked why it was drooping at age 18.

And I remember sitting and listening, because I went down to UCSD mitochondrial research center, and the doctor gave me this 20 minute explanation of what mitochondrial disease was, and I just looked at him and said oh, you mean no two mitochondrial patients are alike, we're like snowflakes, rarely are there two mitochondrial patients that are alike, even in our symptoms presented. He goes yes, that's it.

And I was diagnosed with my mutation, it's a deletion, a primary mitochondrial disease that 14 years ago

fell in the category of Kearns-Sayre syndrome. And I can tell you as somebody that has been heavily involved in reading and with the United Mitochondrial Disease Foundation, boy has the science progressed. I mean it's actually exciting, not to have mitochondrial disease, but the field of mitochondrial medicine.

And so 14 years later I'm told it's more like CPEO+++, which I refer to it like Bud Light. I don't present clinically like a Kearns-Sayre patient, meaning I don't have heart block and pigmentation on my retina, but I have a lot of the other symptoms. And I had a child when I was 35 years old, and when I was 36 years old that's when I was diagnosed.

And I can just tell you from my experience that giving birth and being pregnant was incredibly hard. I think somebody asked that question. And I ended up in a wheelchair after I gave birth. So it took a toll on me, and that was before I knew I had mitochondrial disease, and it was also before my symptoms were as progressed as they are today. And I was in a wheelchair for a while. I'm not in a wheelchair daily now, but I do use assistance if I have to walk distances.

Had I known that I had mitochondrial disease I would not have gotten pregnant. Not that I regret having my son today, but personally I wouldn't have made that choice.

Fourteen years ago I was told I had a sporadic mutation, and 14 years later I'm being told that with people who deal with Kearns-Sayre and that type of deletion that there's a 25 percent chance that my son could have mitochondrial disease. Again, we don't know. We just don't know yet, the science and the research is still advancing.

I guess when I listen to all of the information, and I hope you don't take this in an offensive way, but I was sitting here thinking oh gosh, it would be so great if I was listening to all of this research and it was actually about some therapies for those of us living with mitochondrial disease., helping those of us that our lives are severely affected. But having said that I'm also excited, because my thought was also perhaps this might be the gateway to that. What about those of us living with mitochondrial disease? I've heard numbers, one in 3000, one in 5000, and again those are hard to document for sure.

The other thought I had was if you have mitochondrial disease and problems, I have ten doctors. I'm not a doctor, but I have ten doctors. Healthy people don't go in to check their mitochondria. So by the time you get to the doctor it's because you're having serious problems, and so that was a thought I had. People who have mitochondrial disease are having a hard time getting diagnosed anyway. Fourteen years ago there were seven

centers that could properly diagnose. There was a lot of mis-diagnosis because it took six months. I was negative in my blood but positive in my muscles biopsies. So again, that's another thought I had, was by the time you get to the doctor it's because you're having problems.

And then my thought goes to the women who have mitochondrial disease that are going to be pregnant, and then give birth, and then you live with mitochondrial disease, and then you're taking care of a newborn. And that's a thought that I have, and a concern for those women, and I understand it's an individual choice, but if I was sitting with a group of mitochondrial disease women in their early 20s or 30s I would certainly talk to them on a personal level about that responsibility. It's hard anyway, let alone living with mitochondrial disease. Somebody once said living with mitochondrial disease is like trying to run your house on a battery, and that's what it feels like some days.

And the other thought I had was for my son, that living with a chronically ill parent is something to consider. And he tells me all the time mom, I love you no matter what, but I have to say that for the children living with chronically ill parents, that's just something to consider and think about. That's not something I would wish for a child. You can grow out of that adversity, certainly

there's blessings out of burden, no doubt, but I don't think it's something I would wish for a child if I had that choice.

And then the other thought I had was my dream is for all of the research that's going on with primary mitochondrial disease, if everyone could just get together in a consortium type way. Because I've always said it's not just about those living with mitochondrial disease, but the ramifications and the impact on Alzheimer's and Parkinson's and childhood cancer and metabolic disorders and autism and all the other major diseases that we touch. I don't know that what I've said is helpful, but Gale promised me that I could just speak freely as a mitochondrial patient. So I appreciate that, and thank you.

DR. SNYDER: Thank you very much. Steve?

DR. ROSE: I think most of the previous speakers have said most of what I wanted to say. I think Dr. Lee and Dr. Dahlgren very specifically said we need more numbers in the experiments, and we really need to do micro-array analysis, proteome analysis in these models in order to really understand in a tissue specific way how these defects are being manifested, number one.

Number two I think it gets to what Dr. Moraes said, we're starting off with very low percentages, but as Dr. Winstrom said with time these seem to go up, and we're

not understanding that and we're not detecting it until later in life. And I think we really need to do those long-term follow-up studies, we need to do the biopsies, we need to do the analysis of the genomes, the proteomes, and the micro-arrays in order to really understand how this is affecting the tissue.

I think that Ms. Reeder really made the point, diagnosis, using the animal models to be able to develop better diagnostic tools in order to be able to not have individuals going from doctor to doctor to doctor in order to finally get a diagnosis that means something and is real is extremely important.

DR. GEARHART: I would like to emphasize and concentrate on safety issues, both from a standpoint of what we call epigenetics, and what we see as aneuploidies deriding from these experiments. I think what we're beginning to appreciate, and we're getting some data on this, is how quickly cells can become reprogrammed sitting in a dish.

There is new technology coming, some of us are using it in the lab, where we can convert one cell type to another with the forced expression of a transcription factor of some small molecules. And when you begin these experiments in a dish, it only takes a matter of hours before you see cells change. And it's remarkable, you can

see it morphologically, you can obviously pick it up at the molecular level very quickly.

Now we know, and for the last four or five years, that in a dish pre-implantation stage embryos can undergo changes in their epigenetic program. And it has ramifications downstream. Mainly this comes out of mouse work at this particular point in time. And then couple this with the growing concern in standard IVF practices in humans that these changes will be reflected in adults generated by this process. So it is of great concern to me.

And then when you add to it actual physical manipulation of the cells, I think this could only enhance the likelihood of something like this occurring. Now, the investigators sitting in the first row over there obviously are trying to address this. They're trying to get information on these changes that are occurring, and you heard some reference to it. But we're going to have to get a lot of information. And obviously the mouse may serve as a model for this, because you're going to need numbers. This is one of the things we need, are numbers, numbers, numbers.

I'm sure that the rhesus that have been derived will be analyzed in this fashion too at some point, but it's going to take some time. It's going to take time to generate this information. But this to me, my greatest

concern has unfortunately nothing to do with the mitochondrial disease component of it, it's just how safe is working with these embryos in a dish for whatever period of time that we don't have some untoward outcome. That to me is an extremely important component of this.

The second issue is that of generating aneuploidy, and you can see in small numbers in examples aneuploidy is generated. What's the basis of this? Is it something to do with the spindle, obviously? Do we need more information on what we're actually doing when we do these experiments? Are chromosomes left behind? Because many of these things are missing a chromosome, you don't see an additional chromosome.

And I think we have to gain more information, and I think these investigators are attempting to do that, before we feel some level of confidence that even in the small numbers in which they look at and have been reported that we can somehow reduce this possibility to zero. So to me safety is an issue here for the procedures that are being used in what I think is a legitimate series of diseases to attack.

I want to make one other point, and it's illustrated very nicely for those of you who saw perhaps in the New York Times this morning the report on this meeting. A brief report, but the headlines of this, on designer

babies, conjured up I think the wrong message as to what we are attempting to do at this particular point.

This is something which clobbered us in stem cell biology for years, on and on and on, and we have to do things proactively, prospectively, to get the kind of information out of what our goals are here, and to work with media. This is part of the problem here. I think it has political ramifications on and on and on, and it leads to a complete misunderstanding in the public of what we are about.

DR. SNYDER: I do want to try to get around the table before the break, and focus on what we need for pre-clinical data.

DR. COUTURE: I want to thank all the speakers for some really interesting discussions this morning, and presentations of some of the work in the field. But I also want to say that kind of underscores my view of this thing, I think what we've heard a lot of seminal work, it's not really yet what I would consider to be preponderance of evidence, that this is safe, efficacious, and reproducible.

And I know my comments are similar to John's in that respect, in that I'm really more interested in the safety, but perhaps also as a manufacturer of products, coming from a perspective of reproducibility of the manufacturing process, as it were, it's interesting that in

the cell and gene therapy group, over the 20 years or so I've been interacting with these folks, is we've gone from terminal or near-fatal diseases to less debilitating diseases.

And no we're moving into what would be the opposite end of the spectrum, diseases that don't actually exist, that we're just hoping to prevent the transmission of, and I think that's all appropriate things to talk about. But the risk, benefit, and safety data, that's a three variable type equation that has always been something we've struggled with for 20-some odd years. I think it's changing, it's going to change here as well, particularly in the comments that are made about how we're actually treating here as a future generation.

We talk a lot about heteroplasmy and what it means and understanding what's going to happen in animal studies and to offspring, and I critically want to stress that one piece of the equation is what happens to the firstborn child. To me a much bigger question is what happens after that. I would almost go out on a limb to say I think some of the data suggests we may be able to do this and keep heteroplasmy low in the offspring, but as we just heard that does not in any way predict what's going to happen to the next generation.

We can't do these studies in humans, we can't do

multi-generational studies in humans just to hope that this works out, we're going to have to do some of that in animal models. I would assume the primate model is perhaps the best, maybe the bovine model is an excellent one, I have no idea if the mouse would be actually a good model for that or not. But I think we need much more in both numbers, as has been said by both Dr. Lee and John over here. We need higher numbers, just for the relevance of these kind of studies. We need more reproducibility.

The other piece of this is this also reminds me of kinds of what's happened in transfusion medicine, where bone marrow transplants were fairly straightforward, you took bone marrow out of one patient, you put it into another patient. And the facilities to handle that were really good at what they did, it was a clinical procedure. And then they started getting a little more sophisticated cell selection, and then beyond that we're starting to do genetically engineered cells, it's getting to be fairly complex and complicated.

As those transitions take place so do the testing and whatnot that goes along with those products, and I think a lot of what we're hearing is kind of a background coming from IVF, which is a fantastic opportunity for patients, but it's basically taking what takes place in humans, just putting it in a petri dish and doing a normal

thing, maybe a natural thing. What we're talking about here is quite a bit different than IVF, and I think the testing methodologies as has also been suggested have to come up a bit, I think g-banding, grading of cells is so far lower than what we're going to have to do to understand whether these engineered oocytes are actually safe, has to be developed.

And I'm sorry, just g-banding doesn't cut it. We work a lot with embryonic stem cells, and I can tell you g-banding just doesn't really tell you what's going on in cells, it's just too gross of an analysis, it's just simply karyotype. Having the number of chromosomes is one thing, but actually knowing what's going on in a much more deep dive kind of way is probably going to be important, I think that's been talked about as well.

And the last thing I want to talk about is something I think that has gotten mentioned a number of times here, and I'm no expert in this field, so bear with me, and that is this haplotype mismatch thing. I think we've heard a number of times now, some people think it's a concern, and then the argument is we've had Africans marry Western Europeans, we don't have a problem. But I want to argue that's still a two parent match, and if we already understood the whole threshold effect, et cetera, when you do a three patient mixture you have two unmatched sets of

mitochondria and a nucleus.

I haven’t heard anybody suggest that a two parent mismatch doesn’t have some threshold effect, where one set of mitochondria associated with the nucleus, which could easily overcome any effects of the other set of mitochondria. Myself, I’m just far from convinced that that experience with cross-continental interracial marriages and generating children really bears it all on whether or not there is going to be a safety issue in mis-matching haplotypes in a three parent cross. So I’ll stop there.

DR. SNYDER: Did you have anything else you wanted to add, Dr. Lee?

DR. LEE: I just have a suggestion. It would be good if VA or NIH will have a registry, to collect information from the patients and all the difficult important experiments, because otherwise, like Ms. Reeder said, the importance of information and the experiment are like snowflakes.

DR. SNYDER: So I think after Steve makes his comments we will take a break and do this half of the table right after a 10 minute break, and I promise we’ll adjourn at 5:30 as per the schedule. But I think it’s important for everybody to get their thoughts out.

GOLDMAN: Keeping with the theme of snowflakes, one of the primary points so far today I think has been the

variability in the disease and therefore the difficulty in prognostication without being able to prognosticate with any sort of reliability, developing a clinical therapeutic just isn't going to be feasible. So I'll restrict my comments to what I think would be required in terms of in vitro assessments in terms of the basic cell biology to at least have some better control upon prognostication and prediction.

Much of what I would say in animal modeling has been already discussed. So just to get back to the point of heteroplasmy, clearly there are settings in which it worsens generationally, and settings in which it improves. That speaks to I think at least my fundamental lack of understanding of the nature of heteroplasmy, what are the interactions between mutant and wild type mitochondrial DNAs, and of course the mitochondria that harbor them, how dynamic is that interaction, how dynamic is the competition between those mitochondrial phenotypes within a single cell, how does that competition vary as a function of cell type. As was alluded to a bit earlier, we've heard mostly about what happens in the oocyte, we haven't heard a whole lot about what happens in the differentiated derivatives. And of course as these cells are expanding there's every reason to think that we may see tissue specific differences emerge in terms of the interactions between mutant and wild

type mitochondria.

And so I think all that can be modeled in vitro, and I think those would be a very valuable set of experiments, whether on IPS derivatives from patients or ES as the case may be, but this can all be modeled in defined cell types as well as the pluri-potential cells from which they derives. I suspect it’s going to have to be done on a mutation specific basis, because these interactions may differ tremendously depending on exactly which mutation one is speaking about. So fundamentally I look at that as basic cell biology that to my understanding really remains a hole in the field that we need to fill.

Now, in the setting of that competitive process, if you will, there is certainly mitochondrial stress that is then ensuing. We haven’t really considered or heard much about the apoptosis of the cells that may result from mitochondrial loss, which I don’t think we have considered greatly as a result of that heteroplasmic competition. So whatever is going on in the cell, some of those mitochondria with successive generations are dying, and in that setting potentially inducing the apoptotic cell death of those cells and those lineages. And although the role of mitochondrial signals in triggering apoptosis was discussed by several of the speakers this morning, I think the relationship of that induction of apoptosis to the

interaction between mutant and wild type mitochondria needs to be explored.

We did discuss in the setting of mitochondrial supplementation if you will for infertility the potential effects on redox state. That lends itself to fairly straightforward in vitro experiments, what are the effects now on the different signaling cascades downstream of those changes in cellular redox state, and map kinase dependent pathways controlling proliferation, serine 3 kinase pathways and P-dependent pathways that of course are involved in differentiation.

So proliferation, differentiation as endpoints of given lineages as a function of mitochondrial genotype, I think these are really critical experiments to my mind. In terms of the literature reviewed for today I've seen very little of this as evidence in the literature. But these are fairly straightforward experiments that I think would be very informative to understanding the biology.

In the setting of these types of redox changes, one can see this is a very hot field as many of you know in cancer biology, metabolic reprogramming, that of course imparts epigenetic changes, and those epigenetic changes in the setting of cancers can stabilize the neoplastic state. I would worry about that type of eventuality here as well, in that these cells may be fundamentally altered

epigenetically in the setting of the types of changes in redox state that we may see as a result. Of course, most of these comments are referable to mitochondrial supplementation in IVF. That also lends itself to very discrete experiments in terms of looking at the methylation patterns, the histone modifications of these cells with concurrent measurement of oxidation state.

And again, the tools for these types of studies are readily available, and these are not difficult studies in in vitro models. Here though they'd have to be done in a variety of differentiated lineages, and as a function of individual mutation. That's a point I'd like to close with. Like many of you, I've seen a lot of these patients, and the variability is tremendous.

Without being able to discuss essentially the therapeutic design from the standpoint of individual mutations and individual diseases, looking at all of this in a very generic fashion as mitochondrial disease I don't think will lend itself to clinical trial design, or ultimately to therapeutic development. And so I do think we have to be very specific in terms of identifying which mutations we're dealing with and which cell lineage with every experiment as things proceed.

DR. SNYDER: Thanks a lot. I think what we will do is take a 10 minute break now. I'll reserve my comments to

summarize after the break, we'll do this half of the table after the break. Please be back at 4:15, we'll adjourn at 5:30, and we have three hours tomorrow to mop up with the other questions, so we don't have to finish everything today. See you back at 4:15.

Break 4:04

Resume 4:16

DR. SNYDER: As I said, we will finish at 5:30 today, and we have plenty of time tomorrow to finish the questions. We have nearly three hours tomorrow to be able to flesh out our thoughts and come up with recommendations. So let's just continue around the table, and we'll start with Tabby.

DR. AHSAN: So with discussion topic being pre-clinical studies, it's difficult to address that properly in my mind because there are still some outstanding issues. One of the things is that we've been talking about mitochondrial disease, but it seems like it's either a family of diseases or it's a range, so I will claim that this is not my field, but it seems to be very diverse. So potentially as we move forward with animal studies or preclinical studies we need better granularity on that level. How we would bin it though of course adds other question, but it seems like there needs to be some sort of teasing out of exactly what the question is.

Another issue that seems to be coming up in my mind in terms of the preclinical studies is the evaluation of the raw materials. So we don't seem to have good metrics or quality control or what we would consider to be metrics for quality control on the recipient oocyte, or mitochondrial function. And so those questions are also really important I think as we start to design these preclinical studies moving forward.

And then finally also on the back end, what is it that we believe success to be? There has been a lot of discussion about different aspects of it. Of course in certain studies you could maybe have some sort of in vitro metric, but then whether it's proper development, birth, or even multiple generations of successful progeny. And even there I use the word success without knowing exactly what that means. I think that those are really problematic as we start to try to evaluate preclinical studies. So I raise those issues as things that need to be addressed.

Also, in terms of being a little bit more specific though, I think we really need to increase basic fundamental understanding, potentially maybe through in vitro studies but maybe not. But for example one of the things that the briefing document talked about and I think one other person raised was the spatial distribution of mitochondria within the cells. There was really no

discussion about that. I'm not sure that we understand how function varies depending on that.

There was a little bit of a reference to having to depolymerize actin in order to remove the pronuclei. So there seems to be some aspects of the oocyte that we need to further understand in terms of how that has a consequence in function. Because if the question is what are the inadvertent effects, part of inadvertency or unintended consequences is the fact that we may not be evaluating the metrics that are important to evaluate that. So we really need to increase fundamental understanding on that, as well as maybe the haplotypes.

Another thing that I didn't hear too much about was the relative replication rates of the different mitochondrial DNA. There was some reference in the briefing document about the relative subpopulations varying over time, and so learning more about that and how that could play into it.

And then finally, we didn't really hear much about mitochondrial or cytoplasmic transfer as an approach either, which was also one way to do it. And so thinking about the simplest approach being maybe a good entre, that's one where we're actually changing only the aspect that is deficient. So really furthering understanding on that is really essential.

In terms of animal models, again there has been discussion about which models are best, but of course models have different purposes. So I talked about granularity in terms of what our model would be for the different symptoms of disease, so we may have different models there. But also we have models that clearly are mechanistic. The rodent models help further understanding, and then other models that would be better to predict processes in human. So I think it's important to have clarity on that, that those are not an either or situation but likely an and situation.

And of course in these animal models we come back then to success. So what is the definition of success in this system? And I think in these pre-clinical studies that's also really important, and that takes me back to those three issues about a broad range of diseases, evaluating our raw materials, and having a clear definition of success in order to help inform how we want to design our pre-clinical studies.

DR. SNYDER: Thank you. Dr. Cripe?

DR. CRIPE: I want to begin by acknowledging and expressing my appreciation for the public comments today, which were quite insightful I think and uniformly on the ethical side, which is not really what we're supposed be discussing. Nevertheless I think some of the comments do

bear onto this discussion. Particularly the one that resonated with me is are we really treating a disease or someone's disease, what is the unmet medical need. I think this is a comment that was echoed by our patient representative Sharon today.

And so to me that assessment informs the risk/benefit analysis, and therefore informs where the bar needs to be set for pre-clinical studies. So if there are not any other alternatives for patients to not pass on their mitochondrial disease or not alternatives to the infertility that are acceptable, that's one thing. But if there are alternatives I think that changes the bar a little bit.

So to me the bar needs to be pretty high, because maybe it's insensitive, but adoption is an option, or abstinence, et cetera. But in turning to how high the bar needs to be, I think the in vitro assays, the value of the in vitro studies are really as developing predictive assays and quality control assays, and the question will be then what kinds of assays do these cells need to be put through the paces in order to pass muster.

And it's not clear to me that an assay, I think that's going to have to be tested, what are the different outcomes of those assays and are they predictive of all of the success in an animal model of these cells. SO if

something doesn’t quite pass some evaluation on the heteroplasmy content, is that really predictive or not of failure.

But I think as assurance that this technology is going to be safe the in vitro assays fall far short. It’s very nice and reassuring that cells can differentiate into various types, but that’s not very compelling when it comes to feeling satisfied about the safety overall. So I think as everyone else has said it really is going to take animal models and looking at the successful development to term and perhaps even into later life of offspring. SO to me the bar has to be set pretty high.

DR. EMENS: I would like to also thank the public for their comments, and the patient also for sharing her experiences with us, because that’s very helpful as we consider how to move forward. This is also outside of my immediate area of expertise, and I have to say that I’m coming away from this meeting with a whole new level of respect for mitochondria. Their biology is incredibly complex, they’re a poly-functional cellular organelle. When there are mutations they manifest in heterogeneous ways, their biology seems to be quite fluid, and with the diseases there seems to be variability in the penetrance as well. I think all of that poses a lot of challenges towards translating these types of approaches to the clinic.

So for my perspective I still think we have a lot about the biology to understand, and part of that may reflect my own lack of command of the literature in this area, because it's not my area of expertise. But one of the areas I think is heteroplasmy, and that's been touched on before in terms of an acceptable level of the threshold of heteroplasmy, what types of competition between heterogeneous mitochondria could occur, and even perhaps complementation. I'm still uncomfortable with the whole idea of nuclear mitochondrial incompatibility. There was some very nice data that was shared with us that covered some areas of mitochondrial biology but not all of them, so there are some important areas of mitochondrial biology that were not covered, like apoptosis and things like that.

The other thing that I heard a couple of times that leaves me with some level of discomfort with this is this idea that you could use these technologies to generate a healthy blastocyst, and that does not necessarily translate down the road into a healthy outcome. So I think part of that has to do with the fluidity and the heterogeneity of the biology of the system.

So I think finding ways to characterize the blastocysts in a way to identify those that may turn out to be problematic down the road is something that's going to be very important to do, and the corollary to that is this

need for a long-term follow-up. I agree that we're still early on with our initial experiments in this area and that we really need to follow these studies out, first on a uni-generational level, as well as multigenerational really to understand what can happen here.

DR. BUGBEE: I am taking Doctor Snyder's task to heart and I'm going to focus on our discussion topic. But as a relative layperson, I'm an orthopedic surgeon, did a lot of clinical medicine, and I'm relatively a lay person in reproductive medicine. But I consider myself a relative expert in study design. I do lab studies, small animal, large animal, and clinical studies.

I guess my perspective is that the question at hand here is what do these animal models need to do. In our line of work small animal models I believe are mostly designed for proof of concept, can you do something. And it sounds like from what I've learned today that those have shown that you can do these techniques reliably. Large animal models that have been discussed generally I believe should be focused on safety and efficacy, and in this discussion topic safety is mentioned twice, and the three questions are all about adverse events.

So fundamentally I believe that large animal models are the thing that needs to be focused on before you can answer these three questions about adverse events. Then

I believe you need to perhaps understand what is the relative risk, and we've talked about the bar, what is an acceptable level of adverse events, damage, or bad outcome. And some may say that should be zero, some may say it's five, that's the discussion I believe.

So on that, everything before today maybe I consider a pilot study, and someone could tell me how many primates have been treated, and what is that N. Are there enough studies with enough high quality data that somebody could do a meta-analysis so we could then formulate better questions on all these things? The way I look at this in orthopedics, we've made a lot of mistakes, we usually do things to people, find out they don't work, and then go to the lab. And that's relatively low risk clinical practice, we're not dealing with fatal diseases.

So I wouldn't want us to make that same mistake to try something and then realize wow, the incidence of this is one in 1000, but we only did 100 primates, and so we really didn't study it hard enough. So I think the study design needs to be in a larger animal before we can answer any of these three questions.

DR. CEDARS: So I think this has been a great discussion, and some of this I think has been said, but maybe not in a way that put it together. I think it's really critical for us to develop better diagnostics, not

only for the disease process itself, but as a way to assess this technology. And I think we really need reliable ways to assess mitochondrial content, both DNA and protein, the functionality and the distribution of the mitochondria both before and after the transfer.

I think potentially because somebody mentioned IPS cells, and there are lots of uses for IPS cells in this population, but I think particularly given the variability and the disease process itself that potentially encouraging patients with this underlying disease to donate fibroblasts to do IPS cells to better understand the biology of the mitochondria in this disease and potentially long-term produce gametes that way and understand both the biology of the mitochondria and the disease in that individual person, I think that you really need these testing tools as part of this pre-clinical biology and functionality.

I think there need to be controls for all the manipulations that you do, the reagents and all of that, and you need to go through the entire process without actually doing the nuclear transfer just to see if those manipulations themselves and what kind of effect they have, both genetically and epigenetically. We need to go beyond blast rate, because we know from a lot of studies in the rodent you may get beautiful blasts, you may even get implantation, but you have high reabsorption rates. So you

can damage the embryo but still get a beautiful looking blastocyst.

And then obviously issues of health and delivery. We need to know that there is reproducibility in our assessment of mitochondrial content. This was mentioned in terms of the animal studies. Unfortunately we see a lot in our literature for human studies, is you frequently hear the numerator, but never the denominator.

So there were four monkeys born. How many eggs did that take, how many embryos was that, what's the denominator of all of these studies, not just the success, but what's the denominator? And again, I'm still not sure, knowing the intrinsic risk of mitochondria with aging, that there might not be some negative impact of these manipulations that are going to be exacerbated with aging.

With respect to infertility itself, do we have enough data about normal oocytes in young women, what their mitochondrial content is, and is that assessment reproducible, is it predictive for outcome, and would transfer techniques, and a lot of the techniques in the infertility have been more mitochondrial or cytoplasmic transfer, do you know that you're getting enough mitochondria to reach whatever normal is if we don't know what normal is, and can you assess once you've done these technologies that you've gotten to whatever that level is,

and is there the risk that you'd incur new mitochondrial mutations through these manipulations?

In terms of long-term outcomes, as people have mentioned the different animal models are a little bit of a concern. I mean, the rodent model is helpful because you can go through generations very quickly. There is maze training and things you can do that somehow assess stress or intellectual and neurological capacity, but you'd probably need the primate models for long-term issues of health, metabolic conditions, bonding, cognition, reproduction, and then I think these models need to be stressed, because if you create this model and you keep it in a perfect environment, it would be lovely if we all lived in a perfect environment, but we don't. So I think that these models need to be stressed to see if the mitochondria functionality and the redox potentials and all of that are still in line with what we would consider health for the offspring.

DR. COHEN: I am going to defer to the reproductive biologist on a lot of the issues in discussion topic one. I'm going to have a lot to say tomorrow regarding mitochondrial disease, measurement of mitochondrial function, safety and efficacy, and trial design. Not as a point of argument, but a point of clarification, and I know the FDA doesn't need me to stick

up for them, but the FDA did not approve Thalidomide. The Thalidomide babies born in this country, their mothers traveled across borders to get the medication. Thalidomide was approved after really appropriate safety measures were taken place in 2006. Just a point of clarification.

DR. PERA: It has been a very interesting conversation. When I think about the models, the models are very interesting and they tell us a lot about the biology of reproduction, but I think that there needs to be some human studies done here. There are two things I think that will need to be done. First, I think we need to understand in a woman that has mitochondrial disease does she already have oocytes that are favorable for production of a normal child.

So since we don't know the bottleneck that is in place in segregating mitochondria it seems likely to me that if we're looking at women that are in their 20s and they have for example 20 percent normal mitochondria within a population of 50 oocytes that one might retrieve, and oocyte freezing is possible, and the women are young, that there's probably the ability to screen for oocytes that are already normal.

And so I find that to be just so much more preferable if we look at that very closely than doing spindle transfer, some of the techniques that are being

talked about. So I think that developing a way to screen for embryos that might be normal from women already, using their own oocytes as is, is actually a reasonable approach. One could screen embryos, or one could potentially look at whether or not there's reliability to polar body biopsy.

So I think that in the absence of that type of data, no-one has shown that women do not already have a sub-population of oocytes that are perfectly fine for producing a non-affected individual, I don't think that the science is necessarily merited, and in fact I think it is not merited. It is very interesting science however, and I am a scientist, and I'm very bullish on the science, just not on the human part.

The second thing is, somewhere around the first or second speaker somebody said that perhaps the bottlenecking of mitochondrial DNA in the germ line occurs in primordial germ cells. And we're very well able to make primordial germ cells in a dish, and we can actually understand the process of the bottlenecks that occur on a human mitochondrial genome background. And so I think that I would prefer to look at that if at all possible to begin to see what the frequency is of producing normal germ line from an infected individual.

And then I had a third thing that I just wanted to mention, and I don't mean to offend anybody, but I think

unless we have looked at oocytes in women that are affected, and the bottleneck process in PGC development, if it occurs in PGCs, that we're over-engineering a solution that might already exist.

And so given the ethical considerations, which I do believe actually very valid, although I know that that's not the point of this meeting, but there has been since the birth of Louise Brown a pack that there wouldn't be genetically modified human embryos, and so I think it is a reasonable large deal or big deal to actually go down a different pathway. Now, if there's a need to look at specific mutations we can make IPS lines from people that are affected with mitochondrial disease and look at the segregation of the mitochondria. Thank you.

DR. SNYDER: Thank you. Dr. Bustillo?

DR. BUSTILLO: I come from a clinical setting, I take care of infertile people all day, and I think to answer these questions there are two different groups of patients we're talking about, and I have different feelings in terms of the patients affected with mitochondrial disease and those that are infertile.

I think someone already said that we need to set the bar very high, because there are options to treat both groups of patients that have this. And I agree with Renee here that we should look at the prevalence of normal

oocytes in women who already have mitochondrial disease. If we could diagnose that, or we could diagnose that in an embryo generated by these women, that certainly is a lot more palatable for me in terms of assessment of risk from the manipulations that you're going to carry out with transfer of spindle, et cetera.

So I'm very concerned, my biggest problem when I read all the material and all the literature and listened to the wonderful presentations today, is that I don't know how much data we need to actually assess the safety. I mean how many animal models, in the denominator as Dr. Cedars said. And so I'm very concerned about that, and so I would rather approach it in the other direction and try to figure out other ways with IPS or looking at eggs or something like that before we jump to actual manipulation and putting three different genomes together, et cetera. So that's my feeling.

DR. SNYDER: Thank you very much. Dr. Latham, one of the speakers this morning, wanted to briefly make a comment to clarify some points.

DR. LATHAM: So sitting and listening to the discussion, a really interesting discussion, I guess my concern is somewhere along the way we got really focused on mitochondrial heteroplasmy. I think that's probably especially important when you're talking about the

technique of mitochondrial injection, you're adding mitochondria to the egg. I don't think there's a whole lot of concern about 0.5 percent minority population in heteroplasmy causing severe problems, I would agree with the panelist making that point.

When you're talking about whole genome transfers, spindle transfers, pronuclear transfers, this is oocyte manipulation. There's a fairly extensive body of literature on the adverse epigenetic effects of oocyte and embryo manipulation. So when you're considering those techniques I would just remind you please don't focus on heteroplasmy, don't just focus on the mitochondrial aspects of the cell. Nuclear cytoplasmic compatibility is not just about nuclei talking to mitochondria, it's about the ooplasmic factors acting upon the nuclear genome. So please when you're addressing those manipulations keep in mind that this is an oocyte manipulation, it's not just mitochondrial manipulation, and there is a lot of information out there about that. Thank you.

DR. SNYDER: Just before I summarize, do any of the speakers want to make any brief clarifications or comments?

DR. EGLI: I hear a lot of mentioning of genetic modification, or other aspects of genetic changes. And I think we're all aware that reproduction inherently is a

genetic change, and a sperm in an egg is a genetic change, a tremendous genetic change. And the types of changes introduced or made by this technology has nothing to do with the genetic change done with genetic engineering. We are not having genetically engineered genomes here. These are naturally existing variants of human genomes that are combined.

So the question I think really comes down to what is specific to this technology that does not already exist in human reproduction. And when you look at it it comes down to a few points, which is the manipulation itself, which can introduce some defects in the egg, and I think this is where focus should be, and there are very few compounds like Cytochalasin B, or perhaps the inactivated Sendai virus, that are specific to that technique and not used elsewhere. And I think that’s where the focus should be.

DR. DIMAURO: I hope I had been too successful in convincing everybody about the clinical heterogeneity of mitochondrial diseases. It is true they are very heterogeneous, but mitochondrial diseases are not snowflakes, there are hundreds of patients with MELAS, hundreds of patients with MERRF, well documented, with reasonably consistent clinical picture and well documented mutation. These patients need help.

The women affected, as I showed in two examples, have affected children who die, and have other children who do not die, and see their siblings die. These are the ones we are talking about for this technique. If I came to you with a drug, let's say rapamycin, and convinced you that I can change the heteroplasmy level, I can shift heteroplasmy down below the threshold level, you'd probably consider that very seriously, rigidly would consider of course the toxicity, other drugs that I'm composing, and compare the toxicity with the results.

Well I think what is proposed here is not very different. There are future patients who are going to be seriously or terribly affected by these diseases, and we are proposing what Dr. Egli and others(?) are proposing is to if not wipe out reduce the heteroplasmy level to negligible amounts, like one percent. This is all I wanted to say, because I thought the discussion today was kind of going away from these central points.

DR. SNYDER: What I will try to do for the sake of the FDA is try to summarize very briefly what the sense of the committee is in terms of the differences of opinion, the points of controversy, and the point of agreement. I think that there is no question that there is overall great concern for the wellbeing of these kids, and that some members of the committee wondered whether –-

I think there was a sense of the committee that at this particular point in time there was probably not enough data, either in animals or in vitro, to conclusively move on to human trials based on the pre-clinical data to date, without answering a few additional questions. They felt that these questions were probably more pertinent to mitochondrial disease than to infertility, but the concerns revolved around the pre-clinical data with regard to fundamental translation, but also with regard to the basic science.

So some members of the committee question about the clinical competiveness of mitochondrial manipulation, in other words are there less risky alternatives, have some other avenues such as everything ranging from just adoption to cytoplasmic transfer, and clearly defining what the unmet medical need is. Others felt that the data presented so far preclinically is incredibly intriguing, and in some respects very promising, that in terms of animal models I got the sense that the feeling was that no one animal model would be sufficient.

The bovine was offered as a very nice surrogate for the human because its epigenetics and its folliculogenesis and the way its spindle moves is very reminiscent of the human. The mouse is very appealing because it has very rapid life span, many different

generations that can be followed over time, and there are well established cognitive tests that can be done in the mouse.

The rhesus monkey or a primate is very appealing because it is so like human and can be followed long-term. It was felt by some in the committee that all of these animal models need to be stressed in some way, with provocative maneuvers to see whether their mitochondria or the oocytes after being manipulated can meet the stress.

It was felt by some in the committee that in vitro studies were also very important, that one of the questions would be, both in vitro and in vivo are what does the manipulation itself do, and then do the manipulations directly lead to an improved outcome, and how do we measure success, again whether it’s in vitro or in vivo, that the assays in vitro in particular should not simply be whether an embryo looks good or a blastocyst looks good, but that there should be transcriptomics, genomics, proteomics, epigenomics, looking at the methylene, and that simply looking at a karyotype nowadays is not sufficient.

In addition one would want to look very carefully at mitochondrial function, what is the mitochondrial potential, how does the spindle work, what is this essence of mitochondrial bottleneck, and that agent aneuploidy itself is not sufficient, but looking at what happens to

the mitochondria after these manipulations, and then what happens to the cells whose mitochondria has been manipulated.

For example, looking at their differentiation potential. It was thought that maybe IPS cells, which is a newer technology, has its own inherent variability, may compliment some of this. Even looking at certain manipulations such as cryo-preservation and how that may change the system, or what happens when you introduce for example simply somatic mitochondria DNA into oocytes and see if there's a problem. In terms of looking at long-term, that addresses some of the aspects of carryover, and it was pointed out that sometimes as simple a maneuver as looking at PGD may give a sense of mitochondrial carryover.

In terms of defects in the offspring, it was the sense I got from many in the committee that long-term follow-up was very important, particularly in primates, but also in the mice, and that looking at cognitive function over time was really important, and that function of the animals, again not simply a qualitative judge of what is good or what is bad is very important, but then that raises again the issue of what are the metrics of success, how do we measure success.

There was some initial concern that heteroplasmy was a problem, but then there were others who felt that

when push came to shove heteroplasmy is not really the problem that it would seem to be on the outside based on a lot of animal models to date and in vitro data. There was some concern with regard to these kind of data as regard to what exactly are the sample sizes, have the sample sizes been big enough, and as some of the committee members said, what really is the denominator.

There were some issues also raised, and again this gets down to the issue of clinical/competitive. In other words is this manipulation for a good cause, worth the risk, and some speakers thought that maybe even better diagnostics may be equally or perhaps more important than better treatments, and that we should actually have a better sense of what the patients themselves are, better profiling of the patients, better categorization of the patients, and that maybe some of the studies actually need to be very mutation specific, whether it’s looking at the patients, looking in vitro, looking at the animal models, that for all of these studies standardization of materials across labs is going to be very crucial to be able to compare data cross labs.

I guess the common theme across all there of those questions comes down to safety. Safety, whether it be safety for the embryo, safety for the woman, safety for the offspring. Safety entails looking cross-generationally,

looking at the epigenetics, looking at aneuploidy, looking at the essence of what haploid mismatch really is, even looking at what this does in terms of increasing neoplastic risk, because one changes things, or even changing cell signaling based on redox.

That's my summary of what came out of the meeting. Tell me what I got wrong, what I should add, what I should correct, in terms of bringing closure to question number one, preclinical studies for the FDA.

DR. KEEFE: Just one quick point. As you move into different animal models, the beauty of the two models we heard about today, primate and human, is they're so close to human obviously, and also kind of god created them for spindle transfer. And when we first developed this spindle transfer procedure and published it in Nature Biotechnology in 2000, we had about half a dozen different species.

But the bovine is the most technically challenging because there's a huge amount of birefringent materials, lipids that sit in the cytoplasm, so you have to do all sorts of things. So it's not quite that easy just to start moving it to different animals. These folks have really honed great systems in both the primate and human. It's just a little trickier when you move into another species.

DR. EMENS: The only thing that I didn't hear was

a couple people made comments about the importance of developing consortia.

DR. SNYDER: I had it down in small print. Yes, probably pooling data and having worldwide consortia where people swap and share materials, that was brought out. Thank you.

DR. DIMAURO: Can I say that fortunately there is North American Mitochondrial Disease Consortium, or NAMDC, which puts together right now over a dozen centers of excellent in the United States, for now only one in Canada, hopefully more soon, and in the future in Mexico. And the idea is exactly that, of collaborating, putting together a large series of patients, and defining better diagnostics and looking for therapy, including possibly ooplasmic transfer. But there is a consortium.

DR. SNYDER: I take it you endorse broadening that consortium.

DR. MORAES: Two points. So I think the issue of heteroplasmy again is a little bit overblown, because we have hundreds of publications showing that we know what causes the biochemical defect, it’s the high level of heteroplasmy. So low levels, that is the final readout. We can do biochemistry, but we’ve done it hundreds of times, and it’s always the same.

And the second point I have, I’m a little afraid

that we're not giving good guidance for people working in this field what they should do next, because there are so many things. So maybe we should try to later on be a little more specific in what they should do instead of just saying we need more of everything. Even the model, we're not very clear which ones they should do. I'm just concerned they wouldn't know how to -

DR. SNYDER: I agree, I think being very specific, meaning what readout specifically of mitochondrial function, looking at fission, fusion, segregation, mitochondrial potential.

DR. MORAES: Honestly I think reducing heteroplasmy is a good readout because we already know the biochemical consequences of that. I was more concerned in terms of what they need to do to show safety, to show that the manipulation of these embryos is going to give rise to a normal individual or a normal animal, that's what I meant.

DR. SNYDER: Again showing some success in doing this. I think Dr. Mitalipov brought out that despite his best efforts to actually get patients to donate oocytes with mutated mitochondria, which would address the issue, he's actually had some problems even enlisting patients, and that's obviously incredibly crucial to show that all the manipulation in the end means something.

MS. REEDER: I just think Dr. DiMauro is correct in saying we have NAMDC as a consortium. When I made reference to that I was talking about all of the silos, infertility, cancer, Parkinson's, diabetes, there is research going on in mitochondrial disease in all those different groups, I was talking about a consortium of shared wish list magic wand shared research, not just within NAMDC, but infertility talking to mitochondrial, mitochondrial talking to cancer, those in those groups that are looking at the importance and function of mitochondria, that's what I was meaning, thank you.

DR. SNYDER: So there are things that we can do to help flesh out question number one?

DR. WITTEN: I would like to thank the committee for their exhaustive discussion on this question, and you for that great summary.

DR. SNYDER: We have a half an hour left before we need to adjourn. I think it might be good so that we can use tomorrow productively to begin topic number two. We'll do it in the same way, but I think we'll stop and adjourn at 5:30. I'll read discussion topic number two, and it's much briefer.

Please discuss the potential risks of mitochondrial manipulation technologies to the women with either mitochondrial disease or infertility and to the

resulting children. I think some of this is a recapitulation of what we already talked about, but the new aspect here would actually be risks to the women, and our discussants are going to be David Keefe and Kathryn Winstrom.

DR. KEEFE: I will take a risk here and paraphrase Donald Rumsfeld, 2014, Washington DC. But remember, he said there are things we know, there are things we don't know, and there are things we don't know that we don't know. I think it's again approaching an attack on Afghanistan as you go into this, because you have powerful weapons, and we're not quite sure who the enemy is.

But the things that we know, it's very clear from today's meeting that there is an adept group of creative innovative investigators who can really do nuclear transfer, they can do spindle transfer, pronuclear transfer, and show us they can get excellent embryos in both non-human primates and in humans. That's a big breakthrough and really important, it's a great beginning. And we know from some animal work and some cultural work that there are probably minimal risks of heteroplasmy of neutral genetic variations in the mitochondrial genome.

But then there are the things that we know we don't know. I thought your summary was excellent, there are so many things we don't know. The issues of what about

heteroplasmy of virulent mutations, and what is the cutoff where it becomes an issue. One of the papers that we saw, it's only nine or 12 percent, which is well below the 70 percent required to cause a disease, but in certain conditions, in certain backgrounds, it can progress. So there is a huge list of things that we need to know, and I'll just defer back to the discussion in the animal model, and I think we really should find out those things in animals before we progress to humans.

And then there's that large category of things we don't know we don't know. Beginning with Prometheus, when he brought fire to Earth, we've known for a long time that there's a lot we don't know, and we know that the things are out there, the list is long that the DES brought to us by two very famous state of the art folks from Harvard, a biochemist who was convinced that reproductive failure was from an estrogen deficiency. And the best state of the art material science post World War 2 with the same kind of pressure, we already heard about Thalidomide, and gene engineering, there's a lot we don't know.

And when you enter the domain of humans you have to tread lightly. The issues are especially true with infertility. Most of the discussion today is focused on a very small number of diseased individuals, and I think that's where we should keep the discussion. The idea that

we’re going to do anything to infertility patients involving mitochondria I think should just be off the table.

I think or group was one of the first, along with the group in Colombia, to show that there are deletions in mitochondrial DNA in human oocytes, but these are sort of normal variants, possibly. There’s very little evidence that there’s any oxidative phosphorylation that’s significant for development up to the blastocyst, very little evidence that it makes any difference at all. And of course the patients are very vulnerable, and the data is so lame, I think it really should just not even be discussed. Let’s focus on mitochondrial DNA.

But it does bring up the issue that was discussed earlier about the slippery slope, and this is a very slippery slope when you deal with human reproduction. We must as clinicians but also as scientists balance credulity and skepticism. Patients and subjects put their trust in us. In order to maintain that trust we have to be aware not just of what they want, but of what they need. The razor’s edge between what they want and what they need, what’s the right thing to do? That’s why it’s so critical, the ability to move this even into a select group, we cannot exclude facing the reality that we’re not in UK where there’s a licensed required to do something.

Anybody can start, and just witness what happened with ooplasmic transfer, with no evidence, no control groups, no evidence these folks really had refractory infertility, there are a couple of dozen babies born from this, and we have to be sure that we get this out before we move it out, because we don’t have a license to practice IVF and who can and can’t do this in the US, it’s an entrepreneurial free for all, effectively, although there’s a lot of oversight of this technology.

Risk to the kids, we heard about it. Risk to the mothers, mothers feel the risk to their kids, you can’t separate them. Mothers trust us to do the right thing for them, and having been an obstetrician now for 25 years, so frequently when something goes wrong that very patient turns, you didn’t tell me that cerebral palsy would happen to my child. Everybody here should remember that, even house investigators, the very patient who pleaded and begged and insisted that you push this along is the very one who won’t even look at you, can’t look at you, because she believed you were doing the right thing for her.

And that is going to be the hard thing to know, the importance of knowing when we got it right. When did we succeed? Because the kids are going to be kids. Show me a kid who’s perfect, and that’s going to be the one we’re going to need, because everything else is going to get

blamed on the spindle transfer and the pronuclear transfer.

So to know what we're looking for so that when all the gazillion other things, a little bad in math, disruptive behavior as a teen, a little autism, all these things are going to have to be sorted out. Because we may not think there is competitive evidence that they're caused by this, but that's a very separate discussion than you're going to have with the subjects and the patients who are going to be absolutely convinced, every mother wants the best for her child. I think that's what we're here to discuss, is the best for them.

DR. WENSTROM: I am going to answer the question, but first I want to tell you one of the few pieces of Trivia I know. It was Francis Kelsey at the FDA who kept Thalidomide out of the country, because it had been tested in a variety of rodents and caused no problems, but she was suspicious. So it was one person. So when I think about this question I think about it as a clinician. I think for the risk to the mother initially they are very similar to the risks of IVF, the possibly of ovarian hyper-stimulation, those kinds of things, but those are generally accepted risks.

But I do think this is a very vulnerable population. My experience working with women who have genetic diseases, especially if they think they can have

children, is that they're very vulnerable, and my concern would be how to consent somebody for whom a pregnancy would be very dangerous and might not consider a pregnancy, but then given the opportunity to have this technique might agree to a pregnancy that could actually be life threatening.

It seems to me it would be women with the most severe forms of this condition that would want this treatment. Women with more subtle forms might try to go through pre-implantation diagnosis or some other route. So I think the risk to the mother would be that because of her disease she could be especially vulnerable and it would be very hard to get truly informed consent. Then the pregnancy itself would be risky.

But our patients are adults, if they understand the risks certainly they have the ability to consent to those risks, but I can tell you that pregnancies in women with severe mitochondrial disease can be very risky. The patient I alluded to came in unconscious more than one with hypoglycemia, and her mitochondrial disease was so mild she didn't even have an actual diagnosis name, we just knew it was a mitochondrial defect.

The only other thing I could think for the mother would be if you end up with a triploid conception and somehow diagnose that that could lead to severe

preeclampsia and malignant hypertension, but presumably we would diagnose that because we wouldn't put an aneuploid embryo back in. And then of course the risk of her regret if the baby ends up with problems.

So that leads me to risks for the fetus. We talked about the risk of aneuploidy. I think we established that it probably is increased, although that's one of the few things we can diagnose prenatally, either with pre-implantation, genetic diagnosis, or through the standard prenatal diagnosis techniques, so I don't see that as a huge problem. But I do think there is some evidence in the literature that the mitochondrial function is so important for imprinting, and so much a part of epigenetic changes within the cell, that I would be concerned that the fetus would be at risk for diseases or developmental problems related to aberrant imprinting.

One thing I should have asked that didn't occur to me until I was trying to write this list is that in addition to mitochondrial defects, just a reduced number of mitochondria has been associated with adverse outcomes. So reduced number of mitochondria and mitochondrial depletion has been associated with various forms of cancer, and with imprinting defects because if you don't have enough mitochondria for effective oxidative phosphorylation that affects methylation. So I'd be worried, and I don't know if

that has even been studied here.

But again it comes back to our failure in diagnostic techniques. So it's ironic, the problem that got us here that we can't really do effective prenatal diagnosis for a patient to tell her if one of her embryos is going to be healthy or not is also going to be a big risk in this procedure because it's going to be hard to determine that the resulting children are going to be healthy.

And I don't just mean childhood problems, but even adult onset diseases. Does somehow manipulating the mitochondria lead to mitochondrial depletion, and this child is going to end up dying of cardiomyopathy when it's 40? We don't know and we have no way of diagnosing that or predicting that, so I think it's a big problem.

I think the other risk to the child, and we haven't talked about it, is how to screen the donor for the mitochondria. In one of the references we were given, Taylor and Turnbull, they talked about what was considered a benign variant, the 16189 variant that doesn't even involve a coding region of the mitochondria, that has actually been associated with several late onset multi-factorial disorders, such as cardiomyopathy and type II diabetes. That's something that's considered a normal variant. So what if we used a donor with that variant and

ended up with a child that didn't have mitochondrial disease but ended up with a cardiomyopathy?

So I think we haven't discussed how to screen the donor, and again it gets back to pre-natal diagnostic techniques. so I think that our inability to assure that the children who result from this technique will be normal, not that you can assure that any child will be normal, but that we can't inflicted some new abnormality on this child, that also feeds back and is a big risk for the parents who are undergoing this procedure to have to live with.

DR. SNYDER: Thank you very much. I think probably what we'll do until we hit 5:30 is go around the room again and just make some comments. If the comment that you wanted to make has already been made you don't need to make it again, you can simply pass. But we'll make sure that everybody has a chance to weigh in on this question. Again, this is risks to the women, and risks to the children, and this will be important for building upon tomorrow's topic, which would be what would a first in human clinical trial look like in terms of looking at safety and efficacy issues. So Jane, did you want to add anything?

DR. LEBKOWSKI: Not particularly, I think that those are great comments. One other comment I would ask is, I think it relates to tomorrow's discussion, what are going to be the outcome measures for this particular clinical

trial, and how are we going to be able to distinguish whether any of these potential adverse effects are due to the manipulation or the whole procedure, versus things that would be associated with just normal genetics?

DR. SNYDER: And probably part of the clinical trial should be able to identify these things that we're now calling risks. It should be sensitive enough to flag these if they're going to happen.

DR. DIEKEMA: Just two quick additions, the first of which is when you think about risk in this context you're not just thinking about the risk of the mitochondria manipulation, but also any risks associated with actually being in a clinical trial for who knows how many years. I'm assuming that's going to involve some biopsies and blood draws, and I actually don't know what else, but that has to be rolled into the equation.

And my second brief comment, I'm actually having a difficult time envisioning how I would write a consent form for this. I think the consent process itself poses a risk to these parents. The biggest difficulty I see is that it's difficult to convey to them what their baseline risk is. And if it's difficult to convey that to them it's difficult to convey what the additional incremental comparison risks are, because you don't know what baseline is. And that sets up the potential for real

misunderstanding on the part of the mothers who might participate in this kind of a trial, and real potential for not understanding.

DR. SNYDER: Bonnie?

DR. STEINBOCK: I don't have anything to add to what Kathryn had to say except I'm very glad that parents who aren't in clinical trials don't have to give informed consent to becoming parents.

DR. MORAES: Related to the health of the mother, a concern would be that if they're induced to super ovulate and all that, and they're pretty sick, that might be a problem. I envision a woman doing that if she really wants to have her own genetic kid, probably the father also, so it's a family decision. And probably the best candidates would be like a carrier that is not really sick. So that's probably the best candidate for this kind of procedure. Someone who is really sick, it's a concern to see if the hormonal treatment would then be a problem.

To address some of the other issues relating to donor, the donor I think could be any healthy woman that we sequenced the mitochondria DNA and not find anything that looks pathogenic. I think that would be good enough of a donor for mitochondrial DNA. Risks for the children, I think we discussed that there would be four, but I think that kind of donor would minimize the risk.

Also, the mutation of this particular woman would be well understood. Let's say for example it's the MELAS 3243. So we already know from hundreds of experiments that you need more than 85 percent of that mutation to be sick. So if a woman is going to go through this procedure the mutation should be understood beforehand. And this can be done with fibroblasts or cybrids or things like that, so that you know if you reduce it to one percent the child should be okay.

DR. WOODRUFF: So I will address the risks as might be thought about for Dr. DiMauro's case, which is a young woman who has had two siblings who have succumbed to mitochondrial disease, has low mitochondrial -- The risk would be to the patient that was the index case that Dr. DiMauro indicated, a young woman who had two siblings that had succumbed to mitochondrial disease who was herself young, has low indication for mitochondrial disease herself, and is now seeking advice for what she should do next.

So for example she could have a normal pregnancy, using IVF. There could be the risk of failed IVF in that case. She has the risk of hormone injection, which is ordinary in IVF, and so she could go through a mild or shorter objection as is used for cancer patients. She does have the risk that she would still have an affected child,

even after having gone through substantial intervention with the gametes, both psychologically and physically.

She may have a risk for a pregnancy, so she could use a surrogate. However in this case I think the surrogate consent process would be very difficult because there is the possibility of bearing a disabled child that would have to be either aborted or may have some disability at the outset, so there is that risk. There is the need for genetic counseling, and the requirement for a donor, so I think that we have to think about the risks associated with the donor, and in the index case I would think that you couldn't use a simple donor egg that's come from a donor bank, but rather that individual would have to be consented, would have to be invested in the outcomes, and her mitochondrial DNA would also have to be sequenced to reduce the risk that her mitochondria would have some risk for transmission of new disease that was unaware of in the pedigree of the parent.

There is a risk of cryopreservation. As we heard it's very hard to coordinate both the donor and the recipient in the technology, so there is a non-zero risk associated with cryopreserving gametes. I think those are my risks on the spindle mother, on the donor egg, and on the surrogate potential. All of those represent defend categories on that side of the question.

On the child side there is of course risk of PGD, that's unknown, but non-zero. There is of course the risk that the child will be born healthy and wanted, and that's of course a good risk. There is the risk of the knowledge of the birth. I was at the 30th birthday party for the first IVF offspring in Australia, and there were children who are born with siblings in different media, and they got to the microphone and talked about the psychological issue of the worry of what their siblings' metabolic background might be because of that issue. So the existential risk is non-zero.

There is the risk of health concerns unrelated and related. So they may have an unrelated health issue that didn't come from their conception, but one wouldn't know that. Those that are related represent the risk of an emergence of disease that was within their parentage, but there's also the risk of an emergence of new disease, a new risk that would be associated with the technology, the Sendai fusion itself, the alteration of the microtubules in order to disassemble the spindle in order to move it into another egg.

There's the risk of debilitation while waiting for disease. And we know this from our BRCA carriers, who are very knowledgeable that they carry within their genome the high likelihood that they will have breast or ovarian cancer. These patients by the time they know at 18 or 19 or

20 already are debilitated psychologically in same cases trying to imagine what they're going to do, if they're going to go on a date, at cetera, so these existential risks. There is also the risk if you do go ahead and simply select boys and that boy knows that he was selected and not a sibling of XX lineage.

And then there is the final risk, that these children will grow up and know that they are the link in the middle of an experiment, their mom, and then themselves, and their offspring. These individuals may not themselves want to have offspring, but they will have been part of an experiment since they were born, and they will have some burden of knowledge that there is an expectation of the science that their gametes going forward are part of the experiment, and that would be an additional risk on the part of the child.

MS. REEDER: No further comment on this. I think I commented on number two on number one. That's it for me for today, thank you.

DR. ROSE: I think it has been covered, I think the issue that you brought up about the BRCA1 is a huge one, they're always going to be worried about what's going to happen.

DR. GEARHART: I have no comment about the topic, but I'd like to implore the chair to change the wording in

these questions. It’s just terrible. What it is, it’s oocyte and embryo manipulation. We’re not manipulating mitochondria. I don’t know if people agree with this, but I think if this is printed out this way or published –- I’ll shut up.

DR. SNYDER: That can be part of the recommendations to the FDA, that the questions themselves should be reworded.

DR. STEINBOCK: We all trying to focus on these questions, but there are these underlying conceptual questions that keep coming up about how to characterize this research, Dr. Gearhart and Dr. Egli and members of the public, I think we have to address those conceptual issues.

DR. SNYDER: That could be part of the recommendation. That’s part of our job as well, not only to answer the questions but to pose the right questions, or at least word them the right way. How would you reword question number two?

DR. GEARHART: I think it is very simple. Oocyte and embryo manipulation for the prevention of inherited mitochondrial disease or something like that, and fertility.

DR. MORAES: I was wondering if it’s different from question one.

DR. GEARHART: I think we will come up with

something and recommend it to the chair.

DR. SNYDER: Why don’t you sleep on it. I think we all know what we’re getting at with these questions, and what we can do is sleep on it and get back to me as to the proper way it should be worded. Why don’t we let it slide right now and get to the essence of what we know we’re getting at? The point is well taken, the question should be phrased in a way that is informative not only to scientists but to the lay public.

DR. COUTURE: I won’t be redundant with everything that’s been said, I agree with everything. I will add the one thing though, there’s this presumption, sort of where we’re defining the offspring as part of an experiment, and the presumption is they may or may not approve. I think one of the risks is they may never find out. Things change in kids’ lives, parents pass away, parents move away, maybe don’t want to talk about it 18 years later, and the possibility is we’ll have offspring out there who don’t know they were part of a clinical experiment.

DR. SNYDER: Dr. Lee?

DR. LEE: No further comments, thank you.

GOLDMAN: I agree with all the discrete risks that have been raised. As has been brought up an overarching risk is just that we don’t know what we don’t know. The risk is that we have risks out there we can’t predict,

because the basic mechanisms aren't understood, we don't have any good ability to prognosticate on an individual patient basis. So if we don't know what we should be looking for it can be tough to define what ultimately the risks are and how to quantify outcome once a trial is executed.

DR. SNYDER: We will finish this end of the table and then we will adjourn, because it will be 5:30.

DR. AHSAN: Just very quickly, I think in terms of evaluating risk I agree with everything that's been said. But it's always a risk to benefit ratio that's actually really important to think about as well.

DR. DAHLGREN: No further comments.

DR. SNYDER: With that, I promised that we would adjourn at 5:30, it is 5:30. I look forward to receiving new questions tomorrow. Celia?

DR. WITTEN: Before you close, I just would like to say that in addition to the homework assignment you gave people over night I would just like to say that even though it may be part of what we've said is not explicitly what we're covering at this meeting, I think there's a difference between phrasing the questions and framing the questions, and I'd be interested tomorrow in hearing some comments from professor Steinbach about framing the issues too. I think we would be interested to hear those. Which I

think is not just phrasing.

DR. SNYDER: I will see everybody back here at 8:00 AM. Thank you.

(Whereupon the meeting adjourned at 5:30)