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National Institute of Diabetes and Digestive and Kidney Diseases
New Insights into Congenital Kidney Disease
Virtual Meeting

September 28–29, 2022

DRAFT SUMMARY REPORT

WEDNESDAY, September 28, 2022

Welcome
Robert Star, M.D., National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

Dr. Robert Star, NIDDK, Director, Division of Kidney, Urologic, and Hematologic Diseases, welcomed the participants and remarked that congenital kidney diseases can lead to a range of serious outcomes, including fetal loss and kidney failure in childhood or later. Interruptions to kidney development and low nephron numbers may have implications for lifelong kidney health. Model organism and human genetic studies have provided opportunities to understand the formation of normal and diseased kidneys, but the causes of most congenital renal abnormalities remain unknown. Advances in the past decade have led to crucial discoveries of biology and pathophysiology and insights into key genetic factors and molecular pathways, but important areas remain unexplored. This workshop aims to advance research via discussions with a broad range of experts. Dr. Star pointed out that workshop organizers have sought advice from more advanced disciplines and experts who do not normally consider renal questions in the hopes of identifying new approaches.

Introduction
Susan Mendley, M.D., NIDDK; Eric Brunskill, Ph.D., NIDDK

Dr. Susan Mendley, NIDDK, outlined the workshop's intent to broaden the current research in congenital kidney disease and promote multidisciplinary approaches with diverse expertise and groups of individuals who would not typically collaborate to identify common intersections.

Session One
Moderators: Craig Peters, M.D., The University of Texas (UT) Southwestern Medical Center
Lori O'Brien, Ph.D., The University of North Carolina at Chapel Hill

A Nephrologist's View of Congenital Kidney Disease and Nephron Endowment
Jacqueline Ho, M.D., University of Pittsburgh Medical Center (UPMC) Children's Hospital of Pittsburgh/University of Pittsburgh School of Medicine

Dr. Jacqueline Ho, UPMC Children's Hospital of Pittsburgh and University of Pittsburgh School of Medicine, explained that the nephron is the functional unit of the kidney. Human kidneys contain between 200,000 and 2 million nephrons at birth, with this 10-fold variation formed during normal development. People with a lower number of nephrons are more likely to develop kidney disease. The three-dimensional structure of the kidney is critically important to its function. Normal kidney development begins around the fifth week of gestation and ends around the 36th week, and no nephrons are developed after birth. Kidneys begin developing around the pelvis and later migrate to their final location against the ribcage. Three types of congenital kidney diseases include malformation of the kidney tissue (e.g., absent kidney, small kidney, abnormal kidney), aberrant embryonic migration (i.e., kidneys in the wrong place), and abnormalities in the lower urinary tract, with significant clinical overlap between these areas.

Lower urinary tract anomalies are most commonly unilateral. The presence of protein in the urine and high blood pressure are early markers of kidney disease. If both kidneys are involved in the issue, infants are at risk for poorly developed lungs linked to decreased amniotic fluid, and fetal loss is a risk; many of these infants and small children require dialysis and transplant. Congenital anomalies of the lower urinary tract can be treated surgically, but such treatment may result in irreversible nephron damage, putting children at risk of chronic kidney disease. Persistently elevated creatin is predictive of progressive renal disease, but this biomarker is broad and nonspecific. Congenital anomalies are the cause of kidney failure in about 50 percent of children younger than 4 who require dialysis, which is associated with significant morbidity and mortality.

Infants who are born with one functional kidney have an increased risk of kidney disease later in life, which demonstrates the long latency of congenital kidney anomalies. The results of events that occur *in utero* may not become apparent for 20 to 30 years. One example is multicystic kidney, which results in unilateral kidney hypodysplasia. By the time these individuals reach age 30, they have a 20 percent risk of needing a kidney transplant, but pediatric nephrologists do not follow their patients that long. Dr. Ho emphasized that conditions considered benign in children can result in risks later in life, but defining which individuals have increased risks is challenging.

Nephron endowment, which is thought to help determine the risk of chronic kidney disease and high blood pressure as an adult, is affected by many other features, such as small birth weight, congenital heart disease, and *in utero* exposures and stressors, including environmental exposures and maternal diabetes. The number of nephrons formed in development is influenced by both genetics and environment, but how nephron pattern relates to kidney function, the plasticity of the cells, and how kidneys can be repaired after injury remain unknown. Questions received in the clinic, such as how to recognize and prevent progression of kidney disease, often are challenging to answer.

A Urologist's View of Congenital Kidney Disease
Linda Baker, M.D., UT Southwestern Medical Center

Dr. Linda Baker, UT Southwestern Medical Center, explained that the urinary tract is a living drainage system that is nonresorptive, elastic, and somewhat pressurous. About 0.5 percent of live births have structural urinary tract defects; treatment aims to preserve kidney function, prevent urinary tract infections, and achieve continence at a socially appropriate time, as well as manage secondary effects on embryonic kidney development and optimize urinary tract function.

Dr. Baker outlined some more common urological birth defects, some of which can transduce pressure back onto the nephron and cause kidney damage. Caution is required during surgical correction to avoid affecting the kidneys. Normal urine flow during fetal life is critical to supply amniotic fluid, which the fetus inhales to develop its lungs; fetal obstruction leads to low amniotic fluid and poor lung development and can affect the kidneys.

Prune belly syndrome is a rare disorder that affects mostly male children; affected children have normal cognition but a poor prognosis dependent on lung and genitourinary function. Kidneys may be normal, or their condition may range from mild to major nephrosis. Dr. Baker's team observed that the phenotypic spectrum is broad and created a scoring system to improve phenotyping; they also looked for correlations between genotype and phenotype. One family's history with the syndrome pointed to a mutation in the filament A gene, and most mutations were found in rod 2, the regulatory portion of this protein. Filament A is a crucial mechanosensing, regulatory, and transcriptional protein. Dr. Baker explained that her team believes filament A is in the focal adhesion complex, leading to forced transduction. Other genes were found to cause megacystitis in the cohort, so Dr. Baker's team is challenged to identify how all the mutations integrate.

Dr. Baker outlined her discovery wish list, including a deeper understanding of obstructive uropathies, treatments to guide better fetal management, improved means to assess the functional status of each kidney, and improved discrimination between primary and secondary kidney damage. She also noted the need to increase collaborations between nephrology and urology to improve patient subtyping and increase awareness; NIH-funded workshops could help increase such collaborations.

The Role of Low Nephron Endowment in Disparities in Kidney Disease
Jennifer Charlton, M.D., University of Virginia School of Medicine

Dr. Jennifer Charlton, University of Virginia School of Medicine, explained that although many articles correlate low nephron endowment with low renal function and renal disease, few studies of this correlation have been conducted. People who have too few nephrons to do the work of the kidney are said to have congenital kidney disease. Low nephron endowment is a low nephron mass number at birth; data available on patients with low nephron endowment likely includes those who have experienced nephron loss, such as patients who have been exposed to acute kidney injury, diabetes, nephrotoxins, and obesity.

The few studies using gold-standard techniques to measure nephron number have shown a wide range among humans, with an average of about 1 million nephrons per kidney per individual. Only one such study includes infants, who also show a wide range of glomerular numbers. Glomerular number is lower in those older than 60 years; although many studies have suggested that nephrons are lost with age, no studies have tracked individual patients over time, so this information is unconfirmed. Some studies have shown that males have more nephrons, but data remain inconclusive. One study found that both white and Black participants in the United States and white populations in Australia had similar numbers of nephrons, but native Australians and participants from Japan had fewer nephrons. Dr. Charlton explained the fractionator dissector and acid maceration methods for counting glomeruli and determining nephron number, but neither method allows assessment in individuals, and neither method is translatable.

In mice, knocking out glial cell line–derived neurotrophic factor (GDNF), an important factor in kidney development, results in a lack of kidney development. Animals that are heterozygous for GDNF have a glomerular count that is 30 percent lower than wild type; as they age, these animals develop glomerular hypertrophy and hyperfiltration. *SIRT3* knockout mice also have a reduction in nephrons, with 17 percent fewer than wild type. These animals, when exposed to injury, develop proteinuria and decreased podocyte density, and animals with protein overload had lower survival rates. When ureteral obstruction was induced in an Os/+ mouse with 50 percent fewer nephrons, additional nephron loss was seen even after release of the obstruction. In a model of transplant, animals with a remaining native kidney who received a transplant developed less glomerular sclerosis; animals with only a transplant had a reduced glomerular filtration rate (GFR) and more proteinuria. These studies suggest that having more nephrons or glomeruli is important for avoiding renal disease.

To assess glomerular number in humans, kidneys of donors were biopsied and scanned to determine the cortical volume. Formulas were used to measure glomerular density and estimate the total glomerular number from the cortical volume. The average age of donors was 44, and the average glomerular number was 860, but the range was widely variable across individuals. In another study, glomerular density was assessed in patients who had undergone transplant, and the glomerular number was found to correlate with GFR. In another study, investigators used computed tomography (CT) scans and glomerular numbers to show that people with fewer glomeruli were slower to respond to steroids and achieve remission.

Dr. Charlton pointed out that assumptions have been made in these studies—tissue shrinkage is possible, and cortical volume differs depending on whether imaging is conducted *in vivo* or *ex vivo*. Glomerular density may not be uniform across the kidney. Dr. Charlton's team biopsied kidneys deemed unsuitable

for transplant, infused them with a contrast agent detectable by magnetic resonance imaging (MRI), and identified the total glomerular number, which then could be compared to estimates from biopsies and imaging. Virtual cortical biopsies were conducted in different locations around the kidney. The team determined that more than 200 traditional biopsies would be required to be 95 percent certain that an estimate was within 20 percent of the whole kidney number; four virtual biopsies would be required to achieve this measure, which is an improvement but still not an acceptable amount of invasiveness.

Kidney volume often is used as a surrogate for nephron number, such as when renal hypoplasia is diagnosed by ultrasound, but the range of sizes considered normal is wide. Studies have shown that kidney weight correlates with glomerular number, but in a small range of kidney weights, the range of glomerular numbers remains wide. Dr. Charlton noted that despite remaining questions, such as whether weight correlates to volume and whether the correlation is better in younger people, and the large amount of variation, this measurement is noninvasive and does not involve radiation exposure.

Dr. Charlton noted many remaining gaps in knowledge and pointed out that disentangling nephron endowment and loss is important because solutions for endowment must be defined earlier in development, whereas loss prevention strategies will be implemented later.

Overview of Human Kidney Development
Nils Lindström, Ph.D., University of Southern California

Dr. Nils Lindström, University of Southern California, pointed out that scientists' understanding of the kidney is rudimentary because of its sheer complexity. Nephrons are continuously and asynchronously developing as a fetus grows. Kidney development begins during the first 8 weeks of gestation with the growth of the ureteric bud, an epithelial tube that branches repeatedly during development. Around each tip, pipe cells condense to form the nephrogenic niche, which consists of multiple progenitor cells, including nephron progenitor cells. The formation of nephrons is a gradual and temporally protracted process—nephron progenitors either create more progenitors or become a full nephron, and each cell undergoes this process at a different time. Cells that commit early will become distal nephrons, and those that commit later will become proximal nephrons, creating an axis. As the cells undergo differentiation, they eventually match the cell types in the adult nephron.

Dr. Lindström and his team has worked to assemble a developmental progression of early nephrogenesis and now can depict cellular models. Nephrogenesis progression follows a series of patterns that is very similar between one nephron and the next, suggesting very strict controls for patterning and morphogenesis. Single-cell -omics and three-dimensional modeling have led Dr. Lindström's team to develop a spatial transcriptional map for nephrogenesis, which encouraged them to consider how to connect the early stages of nephron development to adult nephron cell types. Single-cell RNA sequencing (scRNA-seq) allows researchers to assess the expression of genes involved in congenital anomalies of the kidney and urinary tract (CAKUT). When the gene expression in the mouse model is validated in human samples, it is shown to translate very well. Single-nucleotide assay for transposase-accessible chromatin with high-throughput sequencing (snATAC-seq) and chromatin immunoprecipitation sequencing (ChIP-seq) can show expression at the epigenomic and transcription factor level. Comparative *in vivo* studies highlight conserved features in differentiation programs.

Dr. Lindström's team now is working to assemble a gene regulatory network around the cellular decisions that occur during nephrogenesis. The network integrates many modalities, including scRNA-seq, snATAC-seq, bulk RNA-seq, ChIP-seq, and gene knockout experiments, to show the many trajectories cells can take. For example, *HNF1B* could be a node of differentiation for the proximal development pathway, whereas *POU3F3* could be a node for distal tubule cell types. One challenge with pursuing such experiments *in vivo* is the asynchronous nature of nephrogenesis, which makes understanding what genes

do at precise timepoints difficult. Dr. Lindström and his team have developed a system that uses kidney organoid protocols based on induced pluripotent stem cell (iPSC) protocols and biases them toward the nephrogenesis lineage, resulting in 500 nephroids developing at the same time. At about day 12, these nephroids begin to develop a distal or proximal identity. Manipulating *JAG1* dynamics can change the identity of the nephroids to send them to the distal or proximal path. Synthetic cellular organizers are used to modify patterning in the synchronized nephroids, and nephron patterning is controlled by introducing ligand-secreting synthetic cellular organizers. Dr. Lindström suggested that such development maps are a good example of the kind of data researchers can produce.

The Role of Stroma in Renal and Ureteral Development
Thomas Carroll, Ph.D., UT Southwestern Medical Center

Dr. Thomas Carroll, UT Southwestern Medical Center, explained that the renal stroma consists of fibroblasts, mural cells, smooth muscle, and leukocytes. The stroma provides structural support with fibroblasts, the main source of the extracellular matrix, and contractile forces through pericytes, as well as the vascular and epithelial smooth muscle cells that regulate blood flow through the kidney and the peristaltic movements that expel urine into the bladder. The stroma also produces hormones, including erythropoietin and renin; plays an immunogenic role through leukocytes; and produces trophic factors that influence cell growth and survival. In kidney development, the stroma is necessary for ureteric blood branching, nephronic development, collecting duct and ureter development, urinary concentration, nephron formation, mesenchymal–epithelial transition, and vascular formation.

Single-cell analysis identified 17 distinct cell clusters in purified stroma, suggesting as many as 17 molecular subtypes of stroma in the kidney, accounting for its diverse functions. To test this hypothesis, researchers identified differentially expressed genes within each cluster; they also identified a number of expected subtypes, but some cell types remained unidentified, and cortical medullary patterning shows heterogeneity. Dr. Carroll's team has been working to characterize these cell types over developmental time using spatial transcriptomics.

Based on this characterization, stroma types appear to map to all major developmental events during nephrogenesis. Dr. Carroll questioned whether these cells are a reflection of the pattern of the kidney parenchyma or influences on it. Stromal cells can be clustered by transcription factor activity, allowing retrospective studies of previously characterized mice to show which clusters of stroma cells are affected. Using such techniques, researchers identified a lack of fenestrated capillaries in YAP/TAZ mutants, showing that the subpopulation of stroma is necessary for differentiation.

Dr. Carroll summarized that the embryonic stroma plays multiple roles in the development of kidney parenchyma, and the stroma is molecularly heterogeneous. The renal stroma creates unique niches crucial for renal development, differentiation, and regeneration, and defects in stromal differentiation contribute to congenital kidney diseases, including kidney malformations, migration defects, and urinary tract defects.

Discussion with Speakers' Panel

- In response to a question from Dr. Lindström about how often patients are diagnosed with CAKUT rather than a disease with a more precise term, Dr. Ho explained that most of the children she sees are diagnosed via ultrasound and rarely with tissue, so knowledge of structure-function relationships is rare. Individuals with a genetic mutation linked to their CAKUT can be characterized as whole-exome sequencing becomes more available.
- Participants discussed how to improve the ability to phenotype patients and whether the term CAKUT is useful. Dr. Baker pointed out that some people are "lumpers," who prefer to define

broader categories, and some are "splitters," who prefer to use narrower categories. She prefers to split conditions rather than use CAKUT as an umbrella term. Dr. Baker noted that some genes not only alter nephrogenesis but also independently affect maldevelopment of the lower urinary tract, so considering a broader phenotype could lead to greater mechanistic insight. However, by separating phenotypes, subgroups with commonalities can be identified, allowing researchers to study specific mechanisms.

- Participants discussed benefits and challenges of techniques used to differentiate conditions. Dr. Charlton pointed out that biopsies, particularly on abnormal kidneys, already are risky, and sedation of children poses an additional risk. Heterogeneity in a biopsy would make categorization challenging. Dr. Lindström wondered whether imaging can be used, pointing out that no techniques exist to describe how nephrons in a patient are abnormal.
- When asked about potential urinary biomarkers, Dr. Charlton commented that validation is by GFR, which is significantly downstream in children. Better structural biomarkers are needed to improve validation. The time course of congenital kidney disease also is challenging, so pediatric nephrologists need to collaborate with colleagues to follow children into adulthood.
- In response to a question about fetal protein and glomerular fluid, Dr. Baker commented that alpha fetoprotein is made in the liver and could be a confounder of glomerular fluid. Dr. Charlton pointed out that a leaky barrier is possible, noting that premature infants have much higher rates of proteinuria than full-term infants, but markers in the premature patient population are lacking.
- In response to a question about using MRI as a diagnostic tool, Dr. Charlton explained that the contrast agents used by her team are not usable in humans, and sedating children for MRIs is challenging. Her team is working to adapt their technique for other imaging modalities. Dr. Baker pointed out that MRI is used to map the anatomy of the fetus if dilated urinary tract is a concern; she expressed doubts about its level of anatomical detail but noted that it is occasionally helpful. Dr. Craig Peters, UT Southwestern Medical Center, added that MRI can be used to look beyond structure into function. Dr. Baker commented that some literature is available on the measurement of fetal lung volume, which could indicate the quality of fetal parenchyma and suggest whether doctors should intervene to improve predicted postnatal outcomes.
- Dr. Baker provided more information about her study of defective filament A, explaining that her team used CRISPR to create point-mutation mice; when the mouse did not show a strong phenotype, the researchers stressed it with gestational hypoxia, which may be connected to vascular maldevelopment in prune belly syndrome.
- When asked about a formal screening to detect prune belly syndrome early, Dr. Baker noted that her team looked at *HNF1B* but did not find any variants. She is unaware of a screening protocol for hepatoblastoma in prune belly syndrome, which has low incidence. Dr. Meredith Schuh, Cincinnati Children's Hospital Medical Center, pointed out that she has a cohort of CAKUT hepatoblastoma patients and could meet with Dr. Baker to discuss collaboration.
- In response to a question about the relationship of nutritional interventions to nephron number, Dr. Charlton explained that some animal studies show that nutritional modifications can increase the nephron number, but the driving force and long-term outcomes have not been identified. Vitamin A status may be a correlation, but Dr. Charlton was not aware of a relationship between nephron number and weight of the mother. Dr. Ho pointed out that a recent cohort of children of mothers with pregestational or gestational diabetes had an increased risk of chronic kidney disease, but this study did not include a link to renal ultrasounds.
- When asked how nephron progenitor cell timing is determined, Dr. Lindström explained that the answer remains unknown but speculated that cell identity is related to competition for space

within the nephrogenic niche. He added that the initiating factor for new nephrons also is unknown but pointed out that cells that commit early are immediately adjacent to the connecting duct.

- Dr. Deb Gipson, University of Michigan, suggested using data from the Center for Knowledge-Powered Interdisciplinary Data Science (CKIDS).
- When asked if structural MRI would replace biopsies, Dr. Charlton commented that they are complementary, and some information can be acquired from a biopsy but not an MRI.
- In response to a question about what part of the nephron is damaged when aminoglycosides cause nephrotoxicity in neonates, Dr. Charlton explained that aminoglycosides traditionally are considered a proximal tubule toxin. Some data in rats suggest that glomeruli are reduced, so other damage may be possible.
- When asked about differences between human and mouse kidney development, Dr. Lindström explained that the obvious difference is in size and cellularity—in the early stages, humans have twice the number of cells as mice. The distinction between nephron progenitor cells and interstitial cells is very blurred in humans, and gene regulation differs. Arcading is not seen in the early mouse kidney, but causes remain unknown.
- In response to a question about what cellular and molecular processes are actionable in terms of kidney defects, Dr. Peters explained that intervention is possible only in some cases, and indicators for when to intervene are lacking.

Session Two
Moderators: Brian Becknell, M.D., Ph.D., Abigail Wexner Research Institute at Nationwide Children's Hospital
Debbie (Deb) Gipson, M.D., University of Michigan

Parents' Perspective on Congenital Kidney Disease

Ms. Ashley Hart recounted her daughter's medical journey from her diagnosis of bilateral multicystic dysplastic—basically nonfunctional—kidneys. Her daughter required *in utero* treatment to grow lungs in the absence of amniotic fluid, and she started dialysis on her second day of life. After 6 months of hemodialysis at the hospital, she was able to move to home dialysis, and the family now is working to shift from 16 hours of dialysis to 12. The goal is for her daughter to have a kidney transplant at around 2 years old, and her future includes increasing the size of her bladder and working with urology and gynecology specialists. Ms. Hart acknowledged that her family is "standing on the shoulders of giants," and she emphasized the importance of ensuring that her daughter has access to protective factors. She commented on her family's incredible support system, including the thorough research conducted by her medical team. Ms. Hart encouraged attendees to keep working on their research, because what they do matters and mattered to her daughter.

Ms. Nicole Colantuno commented on her son's medical journey. A small piece of tissue blocked the flow of urine *in utero*; by 20 weeks gestation, his kidneys had stopped growing, and from 27 to 36 weeks, he had no measurable amniotic fluid. His lungs ruptured upon his first breath, and he was on breathing support for 13 weeks, with five surgeries conducted during his time in the neonatal intensive care unit. Ms. Colantuno emphasized that none of the doctors they encountered ever told them that he would die. Her son came home 3 months after birth and was on peritoneal dialysis for 10 to 12 hours per day. At 18 months, he received a successful kidney transplant from his father, which changed all the family's routines. He was able to catch up developmentally and start school on time, and he has been stable since the transplant and able to participate in most activities. His health began to decline on the 10th

anniversary of his kidney transplant, so the family currently is involved in the workup for his next transplant. Ms. Colantuno pointed out that the decisions her family made when he was born were "life and death," but now the condition is his experience, and how he feels about it is important. She emphasized the importance of mental health therapy for every member of her family and expressed gratitude for the support of his care teams. She is proudest of her son's resilience and grateful for the perspective this journey has given her, with the understanding that tomorrow is not promised.

Mr. Ted Ferris explained that he was born with congenital kidney disease; by the age of 18, he had had 33 surgeries. He commented that he was blessed to have an amazing care team that did everything they could to support his family. He experienced additional advantages that he recognized not all patients have, such as socioeconomic blessings, a mother who is a pediatric nephrologist, and a healthy family life, and his parents assured him that he was a normal person and that the medical issues did not define him. Mr. Ferris explained that he has had two kidney rejections. The first was "self inflicted" because he did not comply with his medication as a teen; his mother now works on health care transitions to teach pediatric patients how to care for themselves. He emphasized that such programs should be available to all children with pediatric diseases, not just at major institutions. The second rejection was caused by a lack of health care portability; after he moved, his new doctor decided to change his medication without communicating with his prior nephrologist, causing nephrotoxicity. He urged attendees to incorporate time in their calendars to communicate with any new patients' previous doctors. Mr. Ferris commented that health care is designed to manage a disease successfully, but people with chronic diseases also need programs to help them be successful in life. He emphasized the need to look beyond traditional transition programs and "level the playing field" for people who do not have all the advantages he had.

Discussion

- When asked how her family connects to support systems, Ms. Hart emphasized the importance of both individual therapy and marriage therapy, noting that medical trauma is hard on a marriage. She commented that maintaining rhythms and family culture is critical, and she is able to rest in the support of amazing nephrologists. She also pointed out that goodness could be sprinkled into any small moments available during the day. Mr. Ferris added that his parents constantly focused on positivity and taught him the importance of support. Normalizing his situation was a significant driver in who he became, and his parents taught him that tomorrow is always another day. Ms. Colantuno emphasized the importance of turning the situation into something good, such as advocating organ donation and supporting other parents, without questioning why it happened, because the answer does not matter.
- In response to a question about how families feel about participation in studies, Ms. Colantuno explained that her son's nephrologist helped her connect to another parent whose child had a different diagnosis but a similar outcome, and she was able to establish a group of parents through social media and foster connections. She now has friends who are farther ahead in the journey and friends who are newer to the journey, and looking both forward and back is helpful. Mr. Ferris agreed that a support group he participates in as a parent of a child with Down syndrome is essential. As a patient, he valued the realization that he could help other people as he was helped, and he suggested that a more organized process to connect older and younger patients would help patients transition to adulthood.

Session Three
Moderators: Nora Franceschini, M.D., The University of North Carolina at Chapel Hill
Benjamin King, Ph.D., University of Maine

What Can We Learn from the Experience with Congenital Heart Disease? The National Heart, Lung, and Blood Institute Bench to Bassinet Study
Bruce Gelb, M.D., Icahn School of Medicine at Mount Sinai

Dr. Bruce Gelb, Icahn School of Medicine at Mount Sinai, explained that the Bench to Bassinet study started as the Pediatric Heart Network (PHN), a clinical trials network convened by the National Heart, Lung, and Blood Institute (NHLBI). When NHLBI broadened into translational research, the study was developed to span research from basic to clinical. Bench to Bassinet includes three networks: PHN, the Cardiovascular Development Consortium (CvDC), and the Pediatric Cardiac Genomics Consortium (PCGC). The initial requests for application were released in 2008 and funded in 2009 for 6 years, recognizing that these networks would require a full year of planning. Funding for PCGC now is in its third and likely last cycle.

When PCGC began, researchers suspected that congenital heart disease would be genetic—epidemiology suggested a series of linked traits—but whether congenital heart conditions shared causality was unclear, and exome sequencing was costly. The reproductive fitness of any relevant traits would have been evolutionarily low, suggesting the futility of genome-wide association studies (GWAS) for common variants. Additionally, any tissue samples collected, such as through surgical discards, would have been collected a long time after pathogenesis. Phenotyping had cost tradeoffs, so researchers mostly opted for deep cardiac phenotyping and shallow extra-cardiac phenotyping.

The first phase of PCGC emphasized recruitment, assembling the world's largest collection of probands with congenital heart disease for which DNA is available. The cohort includes more than 13,000 people, and full trios are available for more than half the participants. PCGC also has collected nearly 1,500 tissue specimens. Although the cohort has some diversity, it is not optimal, and disease heterogeneity tends toward more severe lesions based on stronger interest.

When PCGC began, about 10 percent of congenital heart disease was believed to be environmental in origin; other known origins included aneuploidy, pathogenic copy number variations, and single-gene disorders. The causes of about 70 percent of conditions, however, were unknown. By 2021, only about 42 percent remained unknown. Dr. Gelb commented that identifying all causes is unlikely, although researchers believe most of the remaining unknown causes are genetic. Despite not yet having identified all causes, PCGC has increased researchers' understanding of single base pair mutations and small indels for both coding and noncoding regions underlying congenital heart disease.

In the early stages of PCGC, the project could afford to sequence only 362 trios, but that allowed researchers to make their first discovery: the role of *de novo* mutations in histone-modifying genes. When the cohort was enlarged, researchers recognized that children born with neurodevelopmental problems and extracardiac anomalies were far more likely to have damaging alterations in genes important for cardiogenesis. When more trios were sequenced, a signal was observed for recessive disorders, and it was correlated to children with isolated congenital heart disease. Dr. Gelb pointed out that the team believes more than 400 genes are involved, of which only 10 percent have been identified.

One lesson learned by PCGC is the importance of cohort size, particularly as technology advances and cost decreases. Initially, the project did not have resources for controls, but they were able to use data on quads collected by the autism community, allowing the use of data on parents and unaffected siblings for controls. PCGC was an early user of genome sequencing; the project was able to find a signal for

noncoding *de novo* variants in congenital heart disease because they could narrow the search space and focus on cardiac-relevant areas. PCGC also arranged some functional assays, but scaling to meet the demands of a discovery project was difficult, and they still are working to increase the numbers. In the last cycle, PCGC aims to increase knowledge of the role of genetic variation not only in causality but also outcomes. To do so, researchers currently are gathering data from electronic medical records. The project also assembled cores to review imaging with the Comparison of Medical Therapy, Pacing, and Defibrillation in Heart Failure (COMPANION) registry. PCGC intends to conduct exome sequencing and single-nucleotide polymorphism (SNP) genotyping on all probands and leave a legacy data set.

Dr. Gelb commented that most of PCGC's work has involved conditions with a high effect size and extremely rare frequency, which mostly are Mendelian and Mendelian-like disorders. GWAS mostly have been conducted in common conditions with low effect sizes, so the space between these types of conditions is largely unexplored and likely involves complex patterns of inheritance. As technology costs continue to decrease, this space can be explored. Only one study has been published that reviews rare variant GWAS; the signal was a missense change in a known heart gene that accounted for 20 percent of the cohort's coarctation of the aorta. This variant is rare, but the penetrance is less than 1 percent. Dr. Gelb pointed out that this is not a Mendelian disease, so the question will be how much such variants underlie congenital heart disease. He noted that the autism research community has shown that low-touch recruitment reduces the cost of recruitment, which enables increased cohort size and discovery, particularly with decreases in the cost of sequencing.

Whole-genome Sequencing (WGS) of Patient Cohorts—The Gabriella Miller Kids First Model
David Higgins, Ph.D., Children's Hospital of Philadelphia

Dr. David Higgins, Children's Hospital of Philadelphia, explained that the Gabriella Miller Kids First Research Act, named after a child who became an outspoken self-advocate for pediatric cancer research after her brain tumor diagnosis, authorized an appropriation of $12.6 million per year for 10 years to the NIH Common Fund for pediatric research. The Gabriella Miller Kids First Pediatric Research Program facilitates research to uncover new insights into the biology of childhood cancer and structural birth defects, including the discovery of shared genetic pathways between these conditions. The Gabriella Miller Kids First Data Resource Center processes and analyzes data sets, develops web platforms to host data sets, and facilitates collaboration and use of data sets.

The program works with clinicians and researchers around the country who are domain experts in their field and have collected samples from consented participants in clinical studies, partnering with sequencing centers to produce high-quality genomic data sets paired with harmonized clinical data. These resources are delivered to the original research group and can be released to secondary researchers later. Data sets released to date include 56,212 files, 13,738 participants, and more than 1.5 petabytes of data with harmonized clinical diagnoses, phenotypes, and demographics. These data include 9,328 structural birth defects studies and 4,410 pediatric cancer studies. Bioinformatics resources include such harmonized genomics files as genomic samples with aligned genomes, trio-based studies with family-based joint-called variants, tumor-normal studies with annotated somatic variants, and RNA-seq studies. These harmonized files have been processed through the same workflow, supporting cross-disease comparisons and analyses. The Kids First Data Resource Portal allows users to explore data sets, build cohorts of participants, discover harmonized genomic data files for further research, and connect data from multiple Kids First studies. The Cavatica platform allows users to compute large-scale workflows on genomic data files, analyze data in the cloud, and share tasks and findings with collaborators around the world.

Dr. Higgins outlined lessons learned while building this data resource. One challenge was organizing clinical data to make integration and cross-analysis as easy as possible. Clinical data vary in breadth and

depth, and all clinical data need review and curation. Ontologies can facilitate cross-study research, and robust data models, such as Fast Healthcare Interoperability Resources (FHIR), support diverse data types. Kids First also has developed tools to receive and ingest data more quickly. The program also has been working to operationalize its concierge quality to produce and deliver high-quality clinical and genomic data sets as rapidly as possible. The workflows are largely consistent across studies within the program, but each project has its own quirks. Effective communication and strong relationships can help ease these challenges, as can effective tracking and project management. Kids First also needs to support its diverse secondary users, who range widely in experience, background, and needs. Maintaining up-to-date references can support interoperability, making the data more valuable. Updating released data sets to the latest reference versions also improves interoperability, although this can require time and funding. The program recently updated its RNA-seq version to support interoperability with the Genotype-Tissue Expression (GTEx) project. Kids First also supports tools that enable use by researchers at every level and robust training, outreach, and support activities, which help increase awareness of the resource.

The Value of WGS of Cohorts in Congenital Kidney Disease
Ali Gharavi, M.D., Columbia University

Dr. Ali Gharavi, Columbia University, explained that genome sequencing is not useful on its own—it must be combined with other data sets, phenotype data, family history data, -omics, tissue samples, computational resources, and data from model systems. At least 600 monogenic causes of kidney disorders are known, with phenotypic overlap in presentation. Some common variants are associated with kidney function, many of which have modest effect sizes. Dr. Gharavi's team used a catalog of monogenic kidney and genitourinary disorders to conduct diagnostic exome sequencing and analysis in a cohort of 3,300 patients with diverse chronic kidney diseases. The diagnostic yield was highest for exome sequencing. A small number of genes accounted for two-thirds of disorders, and many disorders were associated with just a few diagnostic variants, which demonstrates the heterogeneity of diseases and the importance of sample size.

Congenital diseases overlap with cystic diseases, tubulointerstitial diseases, and some copy number variant disorders, which contribute to many malformations, including kidney malformations. Many of these are diagnostic syndromes, and they occur in both adult and pediatric populations. About 4 percent of pediatric patients and 1 percent of adults with congenital kidney disease had these diagnostic disorders. Three common disorders were 17q12 deletion, 16p11.2 microdeletion, and 22q11.2 deletion. These disorders cause a large range of phenotypic malformation, showing the importance of phenotyping. All copy number variant disorders identified in the pediatric population are associated with neuropsychiatric and intellectual disabilities. In one study, patients with one of these disorders scored lower on IQ tests, demonstrated higher levels of anxiety, and scored lower on tests of executive functioning. In another study, an adult cohort with copy number variations scored lower on the Mini-Mental State Examination. These copy number variations have been associated with excess mortality in the UK Biobank.
Dr. Gharavi pointed out the importance of phenotyping for identifying associations. In a phenome-wide association study in the Electronic Medical Records and Genomics (eMERGE) Network, the top signal was for renal phenotypes, followed by neuropsychiatric and skin disorders. Combining electronic health record data with research data was important to identify new disorders.

De novo coding variants account for a large number of congenital defects, suggesting an evolutionary and survival impact. Dr. Gharavi's team assessed trios with renal agenesis, hypoplastic kidneys, or multicystic dysplastic kidneys and found a significant excess of missense and protein-truncating variants in constrained genes and genes for nephron progenitor cells. Dr. Gharavi cautioned that the signal is distributed across a large number of genes, which makes pinpointing a single mutation difficult. Larger sample sizes and more functional studies are needed. Inherited variants also contribute to disease risk, so researchers must combine *de novo* variants, recessive models, and dominantly inherited genes to describe

the genetic architecture of a disease. Incorporating rare variants into the heritability paradigm can add significantly to heritability explanations. Common variants also could be contributing to congenital defects, and whole-exome sequencing shows both common and rare variants.

WGS and large sample sizes also allow researchers to identify noncoding variants associated with risk of disease. Orthogonal data sets are important to reduce the search space in this area. Other classes of mutations that can be sampled include retrotransposons and postzygotic mosaicism, and data can be reviewed iteratively as new information is learned. WGS has some blind spots, such as imperfect structural variation and need for long-read assemblies to fill gaps. Genetic mutations resulting from postzygotic mosaicism can be identified, but only with the right tissues—if only blood is sampled, some such events will be missed.

Overall, WGS has significant value. Most classes of mutations can be detected simultaneously and with higher analytic power than by exome sequencing. WGS also allows researchers to assess the contribution of common and rare variants to architecture. Dr. Gharavi noted that the technique has data management and storage challenges, so harmonized data are important.

Lessons from Discovery of a Monogenic Cause of Disease in about 20 Percent of CAKUT
Friedhelm Hildebrandt, M.D., Boston Children's Hospital/Harvard Medical School

Dr. Friedhelm Hildebrandt, Boston Children's Hospital and Harvard Medical School, explained that five major diagnostic groups cause chronic kidney disease in the first 25 years of life, and CAKUT accounts for about 50 percent. A high number of monogenic disease-causing genes—about 45 currently—have been discovered to cause CAKUT, and about 20 percent of CAKUT patients have been found to have a positive mutation in one of these 45 genes. Monogenic diseases are caused by a mutation in a single gene; recessive genes require both copies to be mutated to cause the disease, and dominant genes require only one copy to be mutated. Of the 45 genes currently known to cause CAKUT, 16 act in a recessive way and 29 in an autosomal dominant way, and different genes may cause similar disease in different patients.

Genetic versus environmental contributions to disease causation occur on a spectrum, but even external causations can have a genetic component, such as susceptibility. In monogenic diseases, the gene mutation is the primary cause, but the phenotype can have other influences. Gene identification allows clinicians to order diagnostics and identify correlations between the genotype and the phenotype, as well as pathogenic mechanisms. Dr. Hildebrandt's team performed whole-exome sequencing on 5,500 families whose members have chronic kidney disease and discovered 10 causes of CAKUT, then studied them in cell biological systems and animal models.

CAKUT is the most frequent birth defect and the most frequent cause of chronic kidney disease in the first 25 years of life. At least 45 causative genes have been discovered to date, and about one in five individuals have a primary monogenic cause of disease, with a high propensity for an autosomal dominant mode of inheritance. More than 200 mouse models have been published, indicating that many more human CAKUT genes have not been identified. CAKUT frequently is syndromic, with external manifestations, and more than 165 syndromic genes are known to have CAKUT as a component. Gene identification has provided insight into at least eight functional models that, if altered, cause a CAKUT phenotype in humans, which can be connected to gene–environment interaction. Genotype–phenotype correlations tend to be extremely weak in CAKUT compared to other monogenetic causes of chronic kidney disease. Allelism can determine whether syndromic involvement is present in CAKUT; Dr. Hildebrandt provided an example showing that different alleles cause different phenotypes with many of the CAKUT genes.

One novel CAKUT gene discovered implicated the retinoic acid signaling pathway in the genesis of CAKUT, suggesting gene–environment interaction. All affected individuals in the study were found to have a stop-coding variation in NRIP1, a ligand-dependent transcriptional co-regulator of nuclear hormone receptors, including retinoic acid receptors, that is recruited by the presence of retinoic acid but acts as a co-repressor. Dr. Hildebrandt's team found that heterozygous *NRIP1* mutation causes an autosomal-dominant form of CAKUT that exemplifies many characteristic features of monogenic CAKUT, including autosomal dominant inheritance, incomplete penetrance, rarity, involvement of transcription factors or co-factors, involvement of the CAKUT signaling pathway, and likely gene–environment interaction. The researchers also showed that *NRIP1* knockout mice replicated the phenotype, and *Xenopus* models replicated the renal morphogenesis phenotype.

Dr. Hildebrandt emphasized that genotype–phenotype correlations are very weak because of variable expressivity, incomplete penetrance, and bilateral dissociation, limiting the impact of prior deep phenotyping on gene discovery and, in some cases, making such phenotyping counterproductive. He noted that CAKUT in humans must have a very strong stochastic component because the same patient often has bilaterally divergent CAKUT phenotypes. Dr. Hildebrandt suggested that reverse phenotyping may be more useful than standard phenotyping, allowing researchers to confirm the specific mutation identified.

Covering All the Bases: Computational and -Omic Resources
Dana Crawford, Ph.D., Case Western Reserve University

Dr. Dana Crawford, Case Western Reserve University, commented on the difficulty of studying the outcomes of congenital kidney diseases, which are common collectively but rare individually, with much phenotypic and genetic heterogeneity. Both inherited and sporadic forms of congenital kidney disease have specific variants of specific disease, as well as copy number variants that can explain the phenotypic outcome, but the genetic basis of the majority remains unknown.

Dr. Crawford outlined computational resources and data types to consider when arranging resources for research. Each consortium has different purposes, but all participants have associated clinical data, biospecimens, and different data types generated for -omic analyses. The most common data type for rare outcomes is whole-exome sequencing data, which is the complete sequence of a coding region of the patient's genome. In the diagnostic setting, this requires two steps: annotation and interpretation. Annotation relies on strong bioinformatics pipelines that draw from existing databases to map the variants to genes and changes that will affect protein function; the data can be filtered to focus on databases with information about variant frequency. Researchers then can interpret the variants as benign or pathogenic. Some resources include ClinVar, which is general, or kidney-specific resources, such as the Polycystic Kidney Disease Foundation variant database.

The community has significant interest in rare variant association studies, but a large number of cases, as well as phenotyped controls without the variant of interest, are needed for studies. Instead of relying on bioinformatic pipelines for such studies, researchers can engage with active areas of research in methods development to determine novel ways to identify association for rare outcomes. One example is a new annotation pipeline that will map variants to the three-dimensional structure of the known protein associated with the gene, which can show distally placed variants that actually are close to each other when the protein is folded, affecting the phenotype. Researchers also are interested in WGS, and changes in technology have enabled cost-effective short-read sequencing across the genome. The recent development of long-read sequencing will support mapping of structural variants for chronic kidney disease without the gaps that have been present in short-read sequences. Long-read sequencing remains expensive, so population-scale data are not yet available. One advantage is that long-read techniques can

sequence native DNA in real time with single-molecule resolution, providing more data on the regulation aspects of the process.

Dr. Crawford advocated continued use of genotyping arrays, which now are very affordable. Pairing cohort genotyping with imputation using the latest reference sequencing can approximate WGS data. Dr. Crawford cautioned that the quality of the data gathered through this approach will vary and depend on the reference panel, actual array, and genetic ancestry of the sample. A genome-wide backbone will allow researchers to contribute to many ongoing GWAS for a variety of traits of interest; GWAS requires a consortium to identify genotype–phenotype correlations. Many downstream analyses are required to determine the cause of a signal. The most common first steps will be *in silico* functional annotation and overlaying the genome-wide signal with an expression quantitative trait locus (eQTL) map to show variants associated with changes in gene expression. A genome-wide background produced with a cheap chip can be used for kidney-specific eQTLs, which can contribute to the scientific community. The most commonly used map for this kind of annotation is from GTEx, consisting of healthy donors with a variety of tissues and a genome-wide background. The downsides of using these maps for kidney research are that the donors were postmortem, only the kidney cortex was sampled, and the data only are in bulk. The Kidney Precision Medicine Project (KPMP) has data from live donors, both healthy people and those affected with chronic kidney disease or acute kidney injury, as well as bulk compartmentalized RNA-seq and heterogenous scRNA-seq data. The project did not generate a genome-wide background, so it cannot yet produce an eQTL map for the community. Dr. Crawford implored attendees to consider creating a genome-wide background early in resource development to provide it as a resource for the entire community and allow investigators to leverage as much data as possible.

Single-cell sequencing also is becoming more popular. Most studies to date have been conducted on bulk and blood samples, so data are not tissue specific. Researchers are interested in obtaining tissue-specific samples, but this will be very invasive for kidney diseases. Single-cell sequencing also can be used to assess subpopulations and somatic variation. Dr. Crawford pointed out that most variation catalogued occurs in the germ line, but if different tissue types are sequenced, they could be correlated with different phenotypes of somatic variation. She also emphasized the need to increase diversity, noting that the genomics community has not prioritized inclusion, resulting in community resources that are heavily European and missing information for other populations. Dr. Crawford added that the lack of diversity is important to keep in mind when recruiting patients and when using external data sets because interpretation will be limited.

Discussion with Speakers' Panel

- When asked about successes in refining congenital heart disease to more homogenous subgroups to identify novel signals, Dr. Gelb commented that success has been limited. He noted that testing multiple hypotheses results in a statistical tradeoff. In many cases, his team has found the gene first, then assessed phenotypes. He clarified that this requires a genome-wide significant signal, which then can be correlated to any specificity in the phenotype. Dr. Gelb noted that these studies sometimes can cross into other disciplines, requiring gene match or other strategies to expand the phenotype. Grouping by phenotype and developing a hypothesis for a genetic commonality is more typical.
- In response to a question about how the congenital heart disease community came together to prioritize areas of study, Dr. Gelb explained that they had been collecting information prospectively. They accepted any information, but the nature of recruitment led to more patients with complex forms of congenital heart disease; in hindsight, these cases were more likely to be found.

- When asked about diversity in relation to annotation, Dr. Crawford explained that data without specific populations would affect results if filters are used that rely on databases for population frequency. For example, some published studies on congenital heart disease suggested that a variant was rare in African American participants based on annotation, but researchers later determined that the variant was common in African American populations and the data were annotated incorrectly. Annotation can change over time based on the data in the database, so reannotating the same data could change the interpretation. Dr. Crawford emphasized that many areas of research are affected by this incomplete understanding of worldwide genetic variation.
- When asked about overlap with other congenital and developmental disorders, Dr. Gelb explained that their first priority is gathering as large a cohort as possible for congenital heart disease to conduct meta-analyses. He pointed out that even the Gabriella Miller Kids First data set has not been used frequently for analysis across birth defects. His team is conducting case control on GWAS, but he cautioned that power decreases depending on the number of trios used, and variation levels are high in noncoding data. He pointed out that trios and *de novo* mutation data are ancestry independent, so researchers have no excuse for a lack of diversity; he added that with case control, good approaches to ancestry are available, but researchers must ensure the data are robust.
- Dr. Higgins pointed out that the Gabriella Miller Kids First data set delivers single individual variants in GCSF format; to facilitate cross-disease analyses, researchers provide the files for each participant, and the investigator can combine them in a joint call to build a cohort. Many investigators ask for a joint call across data sets, but data access and consents limit this. Individuals are more available, and Gabriella Miller Kids First staff try to empower the investigators to decide which to analyze. When asked whether this resource can be used to determine if the data set includes the variant of interest, Dr. Higgins explained that the data are very easy to query on the portal website.
- Participants discussed the inclusion of sequencing data in the KPMP resource in its second cycle, which will be important for increasing the amount of kidney data available.
- When asked about progress related to the *All of Us* Research Program, Dr. Gharavi explained that more than 350,000 participants have been recruited, and 100,000 participants have had genome sequencing; these data are available through the web portal. More than half of *All of Us* participants are underrepresented in biomedical research in some way, so it will be a very useful resource. Kidney disease prevalence is not overrepresented in that population, and pediatric data have not yet been added. Dr. Gharavi theorized that a signal for congenital disorders will be identified when the data have been analyzed.
- In response to a question about gene modifiers, Dr. Hildebrandt commented that in renocystic ciliopathies, many genes encode proteins that generate tight protein complexes. Although most of the genes act in a recessive way, in mouse models heterozygous alleles in components of proteome complexes can act as modifiers. One example in nephrology is the possibility for monogenic recessive diseases to be modified by loci regarding pleiotropy and severity of disease by genes that encode the protein components of these complexes.
- Dr. Gelb commented that pathogenic copy number variants result in variable phenotypes, but some have been associated with short stature. This outcome can be predicted to some extent with polygenic risk scores (PRS), and he suggested that some PRS may apply in the context of renal function. Dr. Gelb noted that the divide between complex and simple genetic causes has been seen as sharp, but that is likely an oversimplification. Dr. Gharavi commented that his studies using the IQ test show correlation with both parental IQ scores and SNP genotype data, so a patient's genetic background might be able to predict whether they will develop kidney disease.

He noted that the common variant space for these congenital diseases has not been explored sufficiently; large effect sizes are frequent but unexpected for common variants, so penetrance is likely incomplete.

- When asked how panelists plan to integrate multiple gene leads to determine causality, Dr. Hildebrandt commented that although a causative variant has been discovered for 20 percent of CAKUT patients, many remain undiscovered, and discovery is limited by the number of patients, so large consortia will be very important. Dr. Gelb reiterated that about 40 percent of congenital heart disease causes remain unexplained; although he anticipates that researchers will be able to identify all genes for *de novo* mutations, the amount of unexplained causes will not change. Oligogenetics is one possible strategy, but statistical power is difficult, and his field has not yet determined whether those variants are sufficient to cause congenital heart disease or simply contributors.
- Dr. Gharavi asked whether researchers are sequencing the right tissue. Dr. Gelb responded that his team has tried sequencing more deeply with few results. He noted that heart tissue must be researched differently than brain tissue, possibly because it is mesoderm derived. The ability to find markers in blood for heart disease may be better than for brain conditions.

Lightning Talks

Dr. Zhe (Zion) Han, University of Maryland School of Medicine, outlined the use of *Drosophila* as a model system for congenital kidney disease. The *Drosophila* filtration structure is highly conserved, so Dr. Han's laboratory developed a series of markers and identified more than 100 kidney-related genes. The *Drosophila* nephrocyte was used to model a novel kidney disease by identifying and silencing the *Nup160* gene; mutations from both parents abolished function in the offspring. *Drosophila* is a powerful genetic modeling system because of its generational speed, low cost, and abundant stock of models available. Dr. Han noted that the kidney driver developed by his laboratory can be used to create kidney-specific gene deletion in a matter of days.

Dr. Gal Finer, Northwestern University Feinberg School of Medicine, explained how her team investigated cross-talk between different cell types using transcription factor 21 (Tcf21), which is required for development of the medullary stroma but shows a marked reduction in kidney stroma. Results suggested that Tcf21 is required for normal differentiation of pericytes and the kidney arterial tree, and a potential role for Tcf21 in enhancing beta-catenin action in stromal cells was suggested. Future directions include assigning a role for Tcf21 in perivascular-endothelial interactions and investigating Tcf21's effects on stromal cell populations.

Dr. Marissa DeFreitas, University of Miami, outlined her laboratory's experiments with neonatal hyperoxia contributions to kidney injury and differential kidney gene expression in rats. Exposure to neonatal hyperoxia was associated with sustained glomerular and tubular injury and increased fibrosis in adult rats at 1 year. These increases were accompanied by a downregulation of kidney developmental gene expression and an upregulation of tubular brush border, extracellular matrix, and antioxidant pathway gene expression. Future studies exploring how antioxidant therapies could mitigate epigenetic changes in kidney development after neonatal hyperoxia exposure are important.

Dr. Anna Strasma, Duke University, discussed pediatric kidney health in agricultural communities affected by chronic kidney disease of unknown etiology (CKDu), which disproportionately affects young male agricultural workers. Although literature suggests that CKDu is caused by workers' exposure to environmental toxins, Dr. Strasma's team proposed that the endemic area affects early life kidney function, with possible additional insult through agricultural work. Kidney injury markers in urine were found at higher levels in the study populations than reference values. Although their current use is limited,

some urinary biomarkers could be used to identify early renal damage in children in endemic CKDu regions. Further research in CKDu endemic communities, including longitudinal studies, will provide greater insights into the role of early life environmental exposures on altered kidney development and susceptibility to kidney disease.

Dr. Robert Chevalier, University of Virginia, explained how his team investigated the variation in nephron number at birth using an evolutionary medicine approach, considering that natural selection is driven by reproductive fitness rather than longevity. Nephrogenesis is regulated by available energy and oxygen. Restricted maternal nutrition favors the fitness of the mother over the fetus; in response to maternal undernutrition or hypoxia, the fetus can reduce its energy consumption by slowed somatic growth or preterm birth. The fetus favors the brain over the kidneys—both consume high amounts of energy, but fetal brain growth is vital to reproductive fitness, and compensatory renal hypertrophy can compensate for moderately reduced nephron number through reproductive years. Although restricted maternal energy signals reduce nephrogenesis, allocating energy to fetal brain growth and favoring reproductive fitness, the tradeoff is an increased risk of chronic kidney disease in adulthood. The global prevalence of these risk factors is 10 to 15 percent of pregnancies, and it is higher in developing countries.

Poster Viewing and Discussion

Participants joined breakout groups to view and discuss the posters presented in the lightning talks.

Session Four
Moderators: Samir El-Dahr, M.D., Tulane University School of Medicine
Ben Fogelgren, Ph.D., University of Hawaii

Using Model Systems to Test Findings from Whole-exome Sequencing
Kameswaran Surendran, Ph.D., Sanford Research

Dr. Kameswaran Surendran, Sanford Research, presented on the use of whole-exome sequencing and model systems to understand the molecular basis of Alagille syndrome (ALGS). He first explained that CAKUT is a grouping of several types of anatomic defects, including regnal agenesis (i.e., no kidneys), renal hypoplasia (i.e., small kidneys), and renal multicystic dysplasia (i.e., abnormally structured kidneys with cysts). CAKUT is a complex disease, and the cause is unknown in most cases. Many monogenic mutations have been associated with CAKUT, but their penetrance is incomplete. Gestational and environmental factors must be considered. Compound mutations also might play a role in the disease.

ALGS is a rare disease that affects multiple organs, including the kidneys. ALGS has been associated with heterozygous mutations in several genes, including *JAG1* and *NOTCH2*. Dr. Surendran explained that not all patients with ALGS develop kidney disease. About 40 percent of individuals with *JAG1* mutations have kidney problems; among these individuals, about 60 percent have small and abnormally structured kidneys with or without cysts, 10 percent have renal tubular acidosis, 8 percent have vesicoureteral reflux, 8 percent have urinary obstruction, and 15 percent have other renal abnormalities (e.g., hydronephrosis, duplex collecting duct system). *NOTCH2* mutations also are associated with multiple phenotypes.

Dr. Surendran outlined two hypotheses that might explain the variable association of kidney disease with ALGS: (1) Only some associated *JAG1* and *NOTCH2* mutations alter cellular processes that occur during kidney development and (2) ALGS-associated mutations in *JAG1* or *NOTCH2* generate a sensitized level of Notch signaling and require mutations in additional renal epithelial Notch signaling network (RENSN) genes to cause kidney disease. Whole-exome sequencing in individuals with ALGS and abnormally developed kidneys resulted in identification of several rare genetic variations (e.g., insertions, deletions,

nonsynonymous SNPs) that are predicted to alter evolutionarily conserved positions within protein-coding regions. Researchers must examine these data further to identify sequence variants that are likely to cause congenital kidney disease.

Model systems are essential for proving causation because human genetic studies can only correlate the occurrence of mutations in a gene with abnormal kidney development. Model systems allow controlled experiments, as well as quick functional characterization of mutants. Dr. Surendran explained that the system must model one or more cellular processes that occur during human kidney development, and the genetic sequence and molecular mechanism of the cellular processes must be conserved between the model and humans.

The mouse provides a useful model for human kidney development, and more than 40 of the monogenic causes of human CAKUT were considered as candidates based on observations in mice. Both the expression of *JAG1* and Notch activity patterns were found to be similar in human and mouse fetal kidneys. Dr. Surendran shared insights from the mouse model. In addition to *JAG1* and *NOTCH2*, other Notch signaling pathway components are required for normal kidney development. Additionally, the occurrence and severity of small multicystic kidney disease increases as the level of Notch signaling is reduced within the developing nephrons. Furthermore, abnormal orientation of epithelial cell division is present in Notch-deficient cells.

Investigators have used cell culture to model these processes. These studies have shown that multiple lumens might arise as a result of abnormal orientation of the mitotic spindle plane. Additionally, Madin-Darby canine kidney (MDCK) cells cultured in a collagen matrix form spherical structures with a single lumen. Inhibition of Notch signaling results in spheres with multiple lumens. Similarly, Notch signaling–deficient cultured kidney epithelial cells have longer primary cilia. Dr. Surendran's group found that expression of ALGS-associated mutant *NOTCH2* in kidney epithelial cells increases cilia length. Eediting of *NOTCH2* also increases cilia length. The group currently is testing this effect in other regions. They also are using CRISPR to induce ALGS-associated *NOTCH2* mutations in mice.

To test the second hypothesis, whole-exome sequencing was performed in a pilot study. The researchers identified 200 to 300 variants of interest per sample. They are determining whether other pathogenic variants are present in RENSN genes in individuals with kidney disease. The investigators are identifying proteins that are proximal to Notch2 within kidney epithelial cells. Several potential variants have been detected in this effort. These findings are being tested further in cell culture and mouse models.

Using Model Systems to Test Findings from WGS
Kristen Brennand, Ph.D., Yale School of Medicine

Dr. Kristen Brennand, Yale School of Medicine, spoke on the use of stem cell models to study the genetic basis of schizophrenia. She explained that the methods developed through her work might be applicable to kidney research. She emphasized that brain disease (e.g., schizophrenia) is common, severe, highly heritable, and genetically complex. Challenges in this area include translating loci to genes to pathways, as well as understanding interactions and summations. Dr. Brennand emphasized that new insights will allow researchers to improve diagnostics, predict clinical trajectories, and discover novel therapeutic targets.

Brain disease can be modeled using human induced pluripotent stem cell (hiPSC) neurons. Dr. Brennand emphasized the importance of considering these systems as disease risk models, rather than disease models. Using this approach, her group explored the effect of genotype on gene expression and neuronal phenotype. The investigators found that manipulation of common variant expression affects function. In follow-up analyses, they reported unexpected synergistic effects between common gene variants.

Dr. Brennand noted that these findings indicate combinatorial perturbations of risk genes that cannot be predicted via one-gene-at-a-time approaches.

Additionally, greater synergistic effects were observed within shared functional sets of genes using RNA sequencing, high-content imaging, and a multi-electrode array. The investigators also found that synergy increases with the number of genes. Genes not detected in the additive model were detected more robustly when examining combinations of genes with shared biological function. Dr. Brennand explained that these effects were found to be "sub-additive" and were enriched for schizophrenia risk. These findings can be explained through convergence.

Dr. Brennand remarked that pooled CRISPR screening is needed to study risk gene convergence at scale. In this approach, the guide RNAs are integrated into the genome, and researchers can determine which guide RNAs are present in various cells (e.g., all cells, each sorted cell marker, each gene signature). Using this approach, her group demonstrated that convergence between common variant gene targets increases with polygenicity. Additionally, eGenes (i.e., genes with an associated expression quantitative trait locus) with shared biological function demonstrated distinct convergent networks: (1) neurogenesis and neuronal activity and (2) secretory pathways and homeostasis. Overall, these convergent genes are enriched for risk genes and drug targets. The group now is using the convergence approach to predict drug response.

Using *Xenopus* Embryos to Model Human Variants
Rachel Miller, Ph.D., UTHealth McGovern Medical School

Dr. Rachel Miller, UTHealth McGovern Medical School, discussed the use of *Xenopus* embryos as a model for congenital kidney anomalies. She began by noting that multiple types of animal models provide value in the context of kidney development. Dr. Miller explained that the frog is an ideal model for the nephron (i.e., the major filtration unit of the kidney) because frogs produce many embryos, which develop externally. Therefore, researchers do not need to dissect the embryo or the kidney. Because the frog kidney develops rapidly, embryos can be manipulated quickly. Additionally, Dr. Miller noted that frog kidney markers have been established, and the epidermis is transparent.

Dr. Miller's group was interested in evaluating conservation of the nephron between frog and mammalian systems. In an analysis of previously collected *in situ* data, they found that nephron patterns were conserved between the groups. In a follow-up study, the investigators evaluated cell types in the frog nephron using scRNA-seq. Cells were grouped into eight major categories based on the expression of selected genes. The investigators then re-clustered the data to compare cell types from frog and mouse nephrons. Using these data, a model was generated to compare the frog and mouse nephron structures, which differ in several ways.

Next, Dr. Miller discussed the application of the model to characterize *DYRK1A*-related intellectual disability syndrome, which has been associated with an increased incidence of urogenital anomalies in some patients. The anomalies appear to be associated with the kinase region of *DYRK1A*, suggesting that kinase activity is important in this disease. Using a frog model, Dr. Miller and her collaborators demonstrated that *DYRK1A* is expressed in the developing kidney. In humans, *DYRK1A* variants were found to disrupt kidney development. Additionally, *DYRK1A* knockdown in frogs disrupts kidney function, leading to edema.

In the future, Dr. Miller plans to further explore the mechanism of abnormal kidney development associated with *DYRK1A* disruption. She also noted that in humans, *DYRK1A* is located on chromosome 21; *DYRK1A* has been associated with disruptions in Down syndrome fetuses that were not carried to term. Dr. Miller is interested in pursuing studies on the regulation of *DYRK1A* levels in kidney

development. Preliminary evidence suggests that overexpression of *DYRK1A* at a high level also leads to disruption of kidney development. Dr. Miller also noted that her collaborators are exploring the association of *DYRK1A* with autism spectrum disorder in a drug screening study.

Last, Dr. Miller noted that the frog also has been used as a model for the neural tube. She explained that myelomeningocele, the most severe form of spina bifida that is compatible with life, constitutes about 50 percent of neural tube defects. Whole-exome sequencing was performed in a group of patients with myelomeningocele to identify and model variants of interest. Dr. Miller remarked that work in this area is ongoing.

Discussion with Speakers' Panel

- When asked whether *JAG1* isoforms are developmentally regulated and whether kidney-associated mutations cluster in specific isoforms, Dr. Surendran responded that *JAG1* is expressed dynamically during development in both mice and humans. He stated that the gene likely plays multiple roles during early nephron development. Additionally, *JAG1* is expressed in various compartments of the adult kidney (e.g., intercalated cells of the kidney collecting duct). Dr. Surendran also noted that he was uncertain about the clustering of point mutations. A clear correlation of the mutation location and disease effects has not been established.
- Dr. Miller stated that several transgenic frog lines have been developed by the *Xenopus* community. A line for the mature nephron utilizes the zebrafish *CDH17* transgenic construct. A more recent line is based on *PAX8*, which also labels kidney progenitor cells. She noted that the community also is developing CRISPR-based tools.
- Dr. Carroll remarked that he has faced difficulties in applying hiPSC models to kidney organoids; the organoids are immature and nonfunctional. Dr. Carroll also noted that transcriptomics analyses will be challenging to apply. Dr. Brennand responded that she has faced similar challenges in neurons. She spoke on the need for more brain cell surface markers, as well as functional assays that can be performed via fluorescence-activated cell sorting. ScRNA-seq is the best solution currently. Dr. Brennand briefly highlighted ongoing work in this area. Dr. Iain Drummond, Mount Desert Island Biological Laboratory, highlighted a recent study using CRISPR screening in human kidney organoids; a robust set of genes was identified. He suggested evaluating the usefulness of that paper within the field.
- Dr. Ben Fogelgren, University of Hawaii, remarked that all the speakers noted the challenge of evaluating hundreds of genetic variants in model systems. He noted that the pooling approach is costly but might be more efficient in the long term.
- Dr. Surendran stated that compound heterozygosity (i.e., for *JAG1* and *NOTCH2*) has not been reported in any patients with ALGS. In response to a question about candidate genes that might be involved in the pathway, Dr. Surendran explained that one gene appears to be mutated consistently, but literature on the function of this gene, in the context of the kidney, is limited. He added that because not all patients with ALGS express kidney disease, mutations in other genes might lead to dysfunction in other organs. Additionally, he remarked that any downstream components of the pathway potentially could play a role. Dr. Surendran pointed out that many signaling pathways, including Notch, initially were characterized in lower-order organisms. Integration of pathways should be considered. Additionally, Dr. Surendran noted that mutations might vary among individuals.
- Dr. Carroll pointed out that for many congenital anomalies, the developmental defects are unknown. Some of these defects might actually be located outside of the kidney. Novel assays are needed to investigate these questions. Additionally, researchers must rely on the identification of multiple mutations in clinical case studies. Dr. Carroll underscored the importance of conducting

more sequencing studies in patients. Dr. Brennand commented that researchers could begin analyzing rare variants of genes to work toward convergence. Dr. Lindström agreed that these studies potentially could be performed at early developmental stages. He commented that studies of later stages, however, likely will be challenging to model. Dr. Lindström added that sequencing studies could capture mutations in enhancer regions, as well as regions that regulate gene expression. Dr. Gharavi agreed that more work in this area is needed.

- Dr. Fogelgren remarked that model systems provide an opportunity to test the interplay among genetic risk factors and environmental influences in congenital kidney diseases.

THURSDAY, September 29, 2022

Overview of Day 1

Eric Brunskill, Ph.D., NIDDK

Dr. Eric Brunskill, NIDDK, summarized the previous day's presentations, noting the quality of discussion and the diversity of topics.

Session Five

Moderators: Carolyn Abitbol, M.D., University of Miami
Lorraine Gudas, Ph.D., Weill Cornell Medicine of Cornell University

Current Approaches to Phenotyping the Intrauterine Environment and Their Limitations

Maisa Feghali, M.D., University of Pittsburgh, Magee-Womens Research Institute

Dr. Maisa Feghali, University of Pittsburgh and Magee-Womens Research Institute, outlined current approaches to phenotyping the intrauterine environment and their limitations. Clinical methods are limited to ultrasonographic assessment of the appearance of the kidney and calculation of amniotic fluid volume; fluid biochemistry can be used in certain circumstances. Use of ultrasound is common during pregnancy because of its safety; most patients receive two or more ultrasounds during their pregnancy. Patients often undergo an ultrasound in the first trimester to assess viability, and another ultrasound sometimes is conducted several weeks later to assess genetic risk. All patients undergo an anatomy ultrasound to survey congenital malformations at approximately 20 weeks. When maternal or fetal risks or malformations are a concern, ultrasounds may be conducted beyond 24 weeks. Many CAKUT abnormalities appear later in pregnancy and can be missed if ultrasounds are not conducted during this time.

Ultrasounds of fetal kidneys can assess their presence and visualize elements of the renal and genitourinary systems, but rarely can assess the complexities of renal function or nephron endowment. Dr. Feghali pointed out that the anomalies most often identified are absence of kidneys, changes in renal epigenicity, presence of cysts, evidence of urinary tract dilation, issues related to keyhole bladder, and changes to amniotic fluid, but these findings are not necessarily indicative of specific diagnoses. Amniotic fluid volume is one of the easiest measurements to conduct, and low or absent fluid often is easily evident. Amniotic fluid volume may be less than expected or absent, but many etiologies are associated with this result. At 20 weeks, amniotic fluid is almost all fetal in origin and can be used as a proxy measure of kidney function. Adequate fluid is critical to normal fetal movement and lung development, and absence of normal renal function and normal source of amniotic fluid in the second half of pregnancy can suggest concerns about neonatal prognosis. Although amniotic fluid volume is technically easy to measure, results can vary widely even day to day and within the same patient because of changes in fluid dynamics; only extreme values are associated with neonatal prognosis.

Amniotic fluid is technically accessible through amniocentesis, which can allow genetic testing and biochemical analysis, but rates of uptake of diagnostic amniocentesis vary significantly. Biochemical analysis has been useful for analyzing lower urinary tract obstruction, but results vary significantly, and multiple samples often are required prior to consideration of interventions or therapies, especially in more advanced cases when guidance about potential therapies is needed. Some studies have suggested that widespread use of serial amniocentesis for kidney diseases indicates poor results; further investigation of these interventions is needed in cases when neonatal prognosis is limited.

Some aspects of maternal physiology and metabolism, including overnutrition, may have a direct effect on the pregnancy environment and the fetuses. A study in Australia found that infants born to obese mothers were smaller than infants born to nonobese mothers. Mouse studies suggest that offspring of mice on high-fat diets have elevated nephron endowments, which could be caused by constituents of the maternal environment that promote nephrogenesis. Both preexisting and gestational diabetes have been associated with increased perinatal morbidity and mortality, mediated by inflammatory cytokines, oxidative stress, and epigenetic pathways, which affect the vascular epithelium of the placenta. Pregestational diabetes is associated with increased risk of metabolic syndrome in offspring, even in the absence of major structural defects classically linked to hyperglycemia. Dr. Feghali pointed out that although this relationship has been identified at the population level, the effect of maternal diabetes on fetal renal development remains largely unknown, although some studies suggest a role for insulin-like growth factors.

Population studies also suggest a relationship between maternal diabetes, hypertension, preeclampsia, and risk of congenital heart disease. A recent study found that infants who had been subject to an impaired maternal fetal environment—including such factors as smoking, hypertension, and diabetes—had a higher risk of death following surgery for single-ventricle heart disease. In preeclampsia, excess placental secretion of SL1 inhibits PLGF and VEGF signaling in the vasculature, resulting in endothelial cell dysfunction. The involvement of these signaling pathways may explain why disorders of placentation in general—and preeclampsia in particular—are strongly associated with fetal congenital heart disease.

Dr. Feghali emphasized that fetal experience is linked inextricably to that of the pregnant mother during a highly plastic period of rapid fetal development, which supports the inclusion of maternal environmental factors and phenotyping during pregnancy in the assessment of congenital kidney development and disease. A preferred clinical outcome or measure to reflect kidney function and long-term risk of chronic kidney disease has not yet been determined, and current evaluation of fetal kidneys is limited in the clinical setting.

Relating Maternal Health and Nutrition during Pregnancy to Fetal Renal Development
Sunder Sims-Lucas, Ph.D., University of Pittsburgh

Dr. Sunder Sims-Lucas, University of Pittsburgh, commented that during his training, studies of nephron endowment often showed that differences in maternal influences reduced nephrons by 20 to 30 percent. He outlined the kidney development process, noting that many structures are conserved in models. Although single-gene mutations frequently are discussed, the causes of more than 50 percent of congenital kidney diseases are unknown and do not seem to be related to specific genes. He hypothesized that many of these conditions are related to maternal factors or influences during pregnancy.

Nephron number is linked to hypertension; a decreased number of nephrons leads to a decreased GFR, tubular enlargement, increased glomerular capillary numbers, increased glomerular sclerosis, and death of glomeruli, which leads to glomerular hypertrophy and hypertension. Poor maternal health leads to a reduction in nephron number and susceptibility to kidney injury and damage. These changes occur regardless of maternal stress, but stresses may cause changes to the functionality of the nephron.

Dr. Sims-Lucas outlined the benefits and drawbacks of current models. Organ cultures are fast and inexpensive and provide clean data related to single genes and single influences on the formation of the kidney, but they do not recapitulate the maternal environment well. This also is true of organoids, which are human-based cells and allow manipulation of simple factors and single genes. Zebrafish have a fast developmental window, support small- and large-scale experiments, are less expensive than mice, and are easy to manipulate genetically. However, they are not mammals, they do not form a permanent kidney, and the models are difficult to relate to the maternal environment. Rodents are mammals and develop all three excretory organs; they have a short gestational period and breed rapidly. They are genetically well mapped, and the maternal environment is relatively easy to manipulate and closely resembles human development. However, rodents are relatively expensive, and kidney development continues postnatally, which is different than humans. Large animal models closely resemble human kidney formation and genetically are very similar to humans, especially nonhuman primate models, but they are extremely expensive and difficult to manipulate.

Some maternal factors that may affect fetal development include nutrition, folic acid levels, vitamin A deficiency, malnutrition, and low amounts of protein. Diabetes leads to reduced nephron numbers and increased risk of chronic kidney disease, and drug use also increases the risk of preterm birth and preeclampsia. Many of these factors lead to reductions in nephron number—such babies look healthy at birth but may be at risk of complications later in life. The global prevalence of diabetes is increasing dramatically; approximately 7 percent of women of childbearing age have diabetes. This prevalence, combined with the prevalence of CAKUT, increases risk of complications. Models are difficult to design. Streptozotocin is a classic model, but these offspring receive known nephrotoxins. A genetic model using the akita mouse, which spontaneously develops diabetes but has wild-type offspring, was compared with unexposed wild-type mice. The diabetes-exposed mice have reduced differentiation; body weight and kidney weight are largely unchanged, but nephron number is reduced by 20 to 30 percent, and they are highly susceptible to kidney injury and salt-sensitive hypertension.

Future research needs include identifying at-risk populations, designing appropriate questions for these populations with the help of epidemiologists, linking maternal stresses to kidney abnormalities, and increasing large-cohort studies. Models for stresses that affect renal outcomes need to be developed and used. Some at-risk populations for maternal diabetes have been identified, but further studies that consider geography and secondary exposures are needed. Existing databases and model systems could be used, and collaborations with basic scientists could be developed to address these questions. Dr. Sims-Lucas emphasized that the ultimate goal is supporting healthy children.

The Effects of Preterm Delivery on Nephron Endowment
Meredith Schuh, M.D., Cincinnati Children's Hospital Medical Center

Dr. Schuh outlined the importance of preterm delivery to nephron endowment. Human nephrogenesis is complete prior to birth, at 34 to 36 weeks gestation. Preterm infants show accelerated glomerular maturation compared to *in utero* development, and the current assumption is that preterm infants undergo postnatal nephrogenesis for no more than 40 days. Preterm infants are at risk for low nephron endowment and therefore have increased risk of chronic kidney disease and end-stage kidney disease later in life. Any infant born before 37 weeks gestation is considered premature. Periviability, at 22 weeks, is the earliest stage of fetal maturity with a reasonable chance of extrauterine survival. An infant born at 24 weeks would ultimately have the nephron endowment of infants born around 29 to 30 weeks. Dr. Schuh provided several examples of studies demonstrating that preterm infants are at much higher risk of developing chronic kidney disease.

Acute kidney injury also affects preterm infants, occurring in about 50 percent of neonates born at less than 29 weeks gestation. Nephrotoxins are prescribed in 90 to 95 percent of neonates born at less than

28 weeks gestation. Although some single-center studies have linked acute kidney injury to renal dysfunction, under-recognition of acute kidney injury and lack of follow-up make studying long-term outcomes difficult.

Additionally, late-gestation kidney development remains poorly understood, and the mouse is not an appropriate model. All mouse nephrogenesis occurs by branching, but human branching nephrogenesis occurs only during the first 15 weeks of gestation. Human kidney development then continues in two additional periods. In the first, arcading, connected nephrons form a chain that drains into a single collecting tubule. An infant who has completed this period would have a nephron endowment of about 200,000. The final period, lateral branch nephrogenesis (LBN), occurs when single nephrons are directly attached to the collecting tubule. All viable preterm infants are born during LBN, which is responsible for more than 60 percent of nephron endowment. Although this period is critical, the specific mechanisms remain unknown.

The hospital course for preterm infants is variable. *In utero*, nephrogenesis continues until 34 weeks, but a baby born preterm will continue nephrogenesis for only 40 days after birth. Before birth, these babies may be exposed to steroids to help lung development; shortly after birth, they may require intubation, mechanical ventilation, antibiotics, fluids, and additional steroids. These treatments will subject the infant to multiple episodes of acute kidney injury that increase the risk for further complications.

Some gaps in knowledge include the mechanisms of LBN and nephrogenesis cessation, whether nephrogenesis occurs normally in the postnatal environment, how treatment of preterm infants affects kidney development, what causes low nephron endowment, and how many nephrons are required for adequate lifelong function. Dr. Schuh showed an example of a study her team conducted in rhesus monkeys, correlated with midgestation human data, to determine that gene expression of nephron progenitor cells decreases with differentiation.

Animal studies have limitations for studying LBN and nephrogenesis in the postnatal environment. Mice, rats, and rabbits continue nephrogenesis postnatally and do not undergo LBN, and primates do not show early cessation of nephrogenesis. Human studies are dependent on autopsy data, resulting in small sample sizes, and are based solely on histological descriptions of nephron development, which may not reflect active nephrogenesis. Human studies also cannot control for the many factors infants are exposed to during a NICU stay. Dr. Schuh noted that although histological studies suggest nephrogenesis ends at 40 chronological days after birth, she has some molecular data showing the potential for ongoing nephrogenesis, suggesting the need to determine whether nephrogenesis continues in some infants and what modifiable factors can be used to continue nephrogenesis in all babies.

Dr. Schuh outlined a study in which preterm baboons exposed to prenatal steroids or transient hyperglycemia exhibited an increased number of mature glomeruli, but those exposed to ibuprofen showed a decreased zone of ongoing nephrogenesis. Accelerated maturation and a decreased nephrogenic zone width also are seen in preterm human autopsy studies, showing that NICU events affect development and appearance, although their effect on nephron function remains unknown. Studies with rats showed differences in nephron number when exposed to indomethacin, ibuprofen, and gentamicin compared to indomethacin alone; although whether these results are medication dependent or model dependent is unknown, they suggest that preterm infants may be more susceptible to nephron loss. Dr. Schuh hoped that Dr. Charlton's future work could further illuminate individual nephron endowment and the requirements for adequate life function.

To summarize, improving nephron number in preterm infants requires models and mechanisms of nephron formation during late gestation. Such a model could be used to study extrauterine kidney

development with varying NICU exposures; both short- and long-term data must be assessed to study nephron number. A mechanism for counting nephron number *in vivo* also is required.

Environmental Chemical Exposures and Kidney Development
Alison Sanders, Ph.D., University of Pittsburgh

Dr. Alison Sanders, University of Pittsburgh, outlined how chemical exposures contribute to the origins of altered kidney development. Exposure to toxic chemicals is a potentially preventable early-life risk factor, and intervention on these risk factors has implications for exposure reduction or elimination, as well as public health protection and clinical practice. Kidney development occurs between weeks 5 and 35; the perinatal period and the maturation period from infancy to early adulthood also is important for kidney function.

Exposure to environmental toxins can occur through a number of routes, including ingestion, inhalation, dermal sources, pesticides, and cigarettes. Environmental exposures also can influence the epigenome. Epigenetic factors are induced by lifestyle and environmental factors, and evidence is increasing that early life environment affects kidney health over the life course. The *in utero* period and the periconceptional period are important windows of susceptibility to exposures for both parents.

Several prebirth and birth cohorts collect environmental exposure information, including the Environmental influences on Child Health Outcomes (ECHO) cohort. ECHO has geographically diverse sites, which is important because exposures are disproportionate across the United States and globally, as well as diverse racial and socioeconomic backgrounds. Many cohorts have been established with a primary focus on neurodevelopment and growth, which can serve as untapped resources on kidney development and larges sources of biobank samples.

A recent review summarized the scant literature on maternal exposures, which showed some consistent relationships between maternal bisphenol A exposures and elevated blood pressure in children, as well as inverse associations between urinary phthalate concentrations and blood pressure in children. The literature for metal exposure is less consistent, with a few studies showing an inverse relationship between elevated maternal lead levels and decreases in such measurements as kidney volume and eGFR. Air pollution is associated with increased blood pressure in children, and a number of emerging nonchemical stressors are concerning, such as heat, humidity, extreme weather, noise, and lack of green space. The health risks of stressors across the lifespan during critical windows of susceptibility are cumulative, and the field is moving toward a better study of mixture effects caused by the many stressors people are exposed to day to day. The kidney's importance as a site of heat-induced disease is particularly relevant in the context of climate change.

Exposure assessment can be challenging; biomarkers are an option, and retrospective analysis often is conducted through questionnaires, but recall bias causes issues. To assess biomarkers at an individual level, longitudinal studies are preferred to capture periconceptional and prenatal exposures. Causality can be a major limitation, but such biomarkers as nails and teeth have some potential for retrospective exposure assessment. The ideal biomarker varies by the chemical of interest—for example, urine is noninvasive and easy to collect, but some metals are not reliably detected in urine.

Some gaps that remain in the field include the need to better define windows of kidney development susceptible to nephrotoxic exposures, improvement of the understanding of risk factors and how they modify the effects of chemical exposures, importance of cumulative factors and gene–environment contributions to susceptibility, and increased understanding of how environmental toxicants influence epigenetics and risk factors.

Discussion with Speakers' Panel

- Dr. Feghali clarified that the ultrasound images in her presentation were not necessarily characteristic of the typical quality, which depends on many factors and may not include sufficient detail to study the kidney. A three-dimensional reconstruction of the kidney is possible with a high-quality ultrasound, but the error rate is about 15 percent. More advanced lesions may be subject to lower error rates, but some uncertainty is unavoidable.
- In response to a question about testing mouse strains with low nephron endowment in parallel with a diabetic model, Dr. Sims-Lucas explained that he and Dr. Ho have studied maternal exposures and identified a 20 to 30 percent reduction in nephron endowment in response to a low-protein diet. The nephrons that form also have a different signature, which suggests that some exposures may change genetic programming. However, he suggested that studying maternal stressors is a better way to assess such changes.
- When asked about her studies of ongoing nephrogenesis, Dr. Schuh cautioned that autopsy data will limit results because of the lack of a functional assay. Classic histological studies confirm that a nephrogenic zone is present; when this zone is not present, markers of nephrogenesis also are lacking. She acknowledged that data are incomplete but hoped the studies could improve the paradigm.
- Participants commented on the importance of three-dimensional reconstructions of the kidney in preventing preterm exposures.
- In response to a question about clinical data included in environmental cohorts, Dr. Sanders pointed out that available data vary by cohort. Birth cohorts usually include clinical data on birth outcomes, often focused on neurodevelopment and growth. When mother and child dyads are followed over time, many clinical measures are collected. Participants suggested contacting the program directors of ECHO regarding opportunities to collaborate.
- When asked about other systems affected by NICU exposures, Dr. Schuh commented that nephrogenesis is unlikely to be the only system affected. Dr. Sims-Lucas added that rodent models show nephrons that look normal, but are lower in number. Patterning is similar between controls and diabetes-exposed rodents, and inflammatory response after injury was small.
- In response to a question about team science–centered awards to gather the type of multidisciplinary expertise shown in this workshop, Dr. Charlton noted that longitudinal studies of children are lacking and would be an important noninvasive way to gather data. One confounder is the large size of kidneys subjected to acute kidney injury. She also noted that clinicians do not necessarily collect kidney data on NICU patients, so such data would not be available in ECHO.
- When asked about models for maternal anemia, Dr. Feghali commented that anemia in isolation does not translate to a major signal, although anemia related to maternal renal insufficiency may have an effect with regard to the background disease. One in three pregnant individuals has some sort of anemia, but researchers have not seen a specific signal in outcomes. Some rodent studies of iron deficiency show differences in glomerular number and increases in fibrosis, but no evidence in humans supports a similar pathway.
- In response to a question about the confounding effects of interoperability on longitudinal follow-up, Dr. Charlton agreed that interoperator variability is an issue. Three metrics are required to estimate kidney volume, and other imaging modalities may be possible, but ultrasound is very easy and patient friendly, especially for NICU patients who cannot undergo extensive imaging. She noted that a sufficiently large cohort probably could overcome some variability issues.

- Participants discussed studies showing that maternal vitamin A deficiency reduces nephron number. Additional studies of vitamin A deficiency in rodents may show effects on lung development.
- When asked if researchers need to consider compensatory hypertrophy when assessing changes in kidney volume, Dr. Schuh agreed that this is important, but the amount of compensatory growth is unknown.

Session Six
Moderators: Michael McMahon, Ph.D., Kennedy Krieger Institute/Johns Hopkins School of Medicine
Jennifer Charlton, M.D., University of Virginia

The Potential Value of Ultrasound Microvessel Imaging in Congenital Kidney Disease
Shigao Chen, Ph.D., Mayo Clinic

Dr. Shigao Chen, Mayo Clinic, spoke on super-resolution microvessel imaging and its potential applications in congenital kidney disease. He first introduced the concepts of resolution—which is determined by point spread function—and penetration in imaging. Dr. Chen explained that in conventional ultrasound, a tradeoff is present between these two parameters. Super-resolution imaging, however, is not dependent on increased frequency. This technique allows users to gain 10-fold resolution without losing penetration. Super-resolution imaging requires the injection of contrast agents (i.e., microbubbles), which can be detected and tracked by ultrasound. A bubble image is generated, and the motion of the bubbles can be tracked to determine flow speed. Optical imaging serves as an independent validation tool.

Dr. Chen presented examples of microvessel images and velocimetry maps generated using this technique. He shared representative data generated in rabbit and human kidneys, highlighting examples of changes in morphology associated with chronic kidney disease. He explained how motion registration enables alignment of the image. The bubbles then are localized and cumulated. He also highlighted separation of arteriole and venule images, which is used to characterize the microvasculature. A total chronicity score can be calculated.

He also explained that microvessel imaging can be performed without a contrast agent. This alternative offers benefits in certain cases. Power and color dopplers can be applied for microvessel imaging. Dr. Chen shared representative data in rat liver, rat kidney, fetal rat heart and lung, and adult human kidney. He noted that the penetration and resolution tradeoff effect is apparent, but the benefits of not using a contrast agent warrant further exploration of this approach.

Potential applications of vessel morphology maps in kidney disease include visualization of kidney injury; disease detection, staging, and treatment monitoring by studying microvascular rarefaction; and correlating vessel density with nephron number for prognosis and follow-up. Potential applications of flow-speed maps include detection of hyperfiltration, as well as studies of flow change before and after stimulus in the context of renal functional reserve.

Radiologic Approaches to Phenotyping
Sila Kurugol, Ph.D., Boston Children's Hospital/Harvard Medical School

Dr. Sila Kurugol, Boston Children's Hospital and Harvard Medical School, discussed the use of MRI for phenotyping congenital kidney disease. She began by explaining that MRI is an ideal approach for characterizing kidney morphology and function. Functional changes due to disease precede anatomical changes, and functional MRI could enable early detection and improve understanding of disease

pathogenesis. One challenge associated with MRI is motion, which can reduce the robustness of the approach. The goal of her work was to improve imaging to generate robust quantitative markers.

Ultrasound often is used for imaging congenital kidney disease both prenatally (i.e., for detection) and postnatally (i.e., to confirm diagnosis and define the defect). MRI can be used prenatally and postnatally for qualitative evaluation (e.g., anatomy, function) and for quantitative imaging of markers for renal injury, microstructure, and function. Postnatal MRI is used for anatomic and functional evaluation (e.g., GFR, differential renal function, perfusion). Dr. Kurugol shared representative data generated using these techniques. She briefly outlined the process for determining GFR and noted that volume might not be a sufficient marker for this measurement.

These techniques have been applied for use in human infants. The infant is fed, put to sleep (i.e., without sedation), and placed in the scanner. Dr. Kurugol noted that motion—which can lead to signal dropout and misalignment—remains a challenge for accurate GFR estimation. Motion-corrected dynamic contrast-enhanced MRI, however, can be used to realign images and improve the quality of the measurements.

Dr. Kurugol also highlighted non-contrast MRI techniques. Diffusion-weighted MRI (DWI) can be used to detect early changes in microstructure, as well as renal injury and fibrosis. Dr. Kurugol shared representative data generated using this technique. DWI can provide imaging markers for renal disease, and contrast is manipulated by changing the encoding diffusion. Motion remains a challenge; slice-to volume registration, however, can help researchers overcome this limitation. Geometric distortion also can present an issue and necessitates corrective measures.

Additional techniques include spin labeling, which determines perfusion through endogenous blood labeling. Relaxometry can be used to determine tissue characteristics and identify cysts versus tissue. Blood oxygenation level–dependent imaging can be used for renal oxygenation measurement of deoxyhemoglobin. Compared to fetal ultrasound, fetal MRIs provide better soft tissue contrast, as well as a wide range of tissue contrast. Different MRI techniques offer various benefits for structural and functional characterization. Dr. Kurugol shared an example of the use of motion-robust DWI for fetal imaging.

In conclusion, MRI can provide both anatomical and functional evaluation. Multi-parametric MRI can provide multiple quantitative markers of kidney function, renal perfusion, oxygenation, and changes in tissue microstructure. Obtaining reliable and robust quantitative imaging markers will require improved methods for motion and artifact correction, as well as standardized MRI protocols. Validation studies are needed to evaluate markers for detecting early changes in function and microstructure due to renal injury and defects.

Developing a Cohort Study—Lessons from the Epilepsy Phenome and Genome Project and Epi4K
Heather Mefford, M.D., Ph.D., St. Jude Children's Research Hospital
Ann Poduri, M.D., M.P.H., Boston Children's Hospital/Harvard Medical School

Dr. Heather Mefford, St. Jude Children's Research Hospital, and Dr. Ann Poduri, Boston Children's Hospital and Harvard Medical School, presented on the use of cohort approaches for genomics studies that can provide new insights into congenital kidney disease. They first provided an overview of epilepsy, which is defined as recurrent, unprovoked seizures. Epilepsy is highly common, with a 1 in 26 lifetime prevalence. Morbidity results from seizure and non-seizure symptoms, and the disease is associated with an increased risk of mortality. About one-third of patients with epilepsy do not respond to treatment.

MRI has provided new insights into structural abnormalities seen with epilepsy, which vary markedly among patients. Epilepsy is known to have a genetic basis, and many types of epilepsy exist (e.g., generalized epilepsies, focal epilepsies, development epilepsies, epileptic encephalopathy), possibly resulting from different causes. Genetic studies can provide insights into the underlying causes of different types of epilepsy. The Epilepsy Phenome/Genome Project, a cohort-based effort, was initiated in 2007 to explore this topic further.

Genomic studies were later enabled with the development of next-generation sequencing, which provided an efficient and cost-effective approach for this effort. Researchers sequenced many genes simultaneously and pursued hypothesis-free discovery. The Epi4K Consortium was established to apply novel sequences approaches to a patient cohort. During the past 10 years, recent advancements in sequencing technologies have enabled the identification of many additional genes associated with epilepsy.

Epi4K's early guiding principles include strategy and stakeholder input, clear structure and guidelines, publications guidelines (e.g., authorship, primary vs. secondary papers, data sharing), and career development focus. Benefits of Epi4K include scientific success, collaborations, data sharing, accelerated discovery, rigor (e.g., phenotyping, genomics, statistical genomics), and networking. Other considerations include compromises (e.g., prioritization of phenotypes, prioritization of analyses, timing of publications, personalities) and individual versus consortium work (e.g., credits to junior investigators, priorities, follow-up studies).

Discussion with Speakers' Panel

- Dr. Chen explained that the resolution associated with the super-resolution imaging technique is 50 microns, and vessel count represents a critical question in the field. Vessel counts are feasible, but reliability of this metric is a concern.
- When asked whether the heterogeneity of vessel density can be determined, Dr. Chen responded that three-dimensional structures will be needed to address this topic. He noted that he is performing pilot studies in this area. Three-dimensional imaging can help with addressing motion-related issues.
- Dr. Chen noted that he has not yet performed unilateral analyses. He currently is focused on improving the reliability and robustness of super-resolution imaging, which still is in early development.
- In response to a question about the use of using T1rho relaxation time sequence in kidney MRI, Dr. Kurugol noted that she has not yet used this approach but plans to pursue various approaches in future studies.
- When asked about the use of radial imaging to improve MRI measurements, Dr. Kurugol replied that advancements in this area have enabled the generation of higher-quality and motion-robust images. The approach now can be applied in newborn infants.
- Dr. Charlton remarked that genetic diagnoses often do not agree with researchers' perceptions of the phenome. She wondered about categorization of the phenome. Dr. Poduri explained that most characterizations initially were based on generated hypotheses. She noted the complexity of epilepsy classifications within the field and emphasized the need to establish meaningful phenotypes. Dr. Mefford added that detailed phenotype data can help explain genetic findings.
- When asked about the use of electronic health records (EHRs) to characterize phenotypic presentation. Dr. Mefford noted that errors in medical records can pose an issue for researchers. EHRs could, however, be used for initial identification of patients. Dr. Poduri added that such data-mining efforts have shown mixed results. She noted that clinical inputs often are not

standardized. A centralized database would be beneficial, but development of such a resource has been challenging.

- In response to a question from Dr. Charlton, Dr. Poduri clarified that the study enrolled presumed genetic, non-acquired epilepsy patients. Other studies have examined acquired cases in preliminary studies, and several unexpected findings have been reported. Dr. Mefford added that genetic predisposition is likely to play a role in those cases, but the basis might be more challenging to determine. Many factors must be considered.

Breakout Sessions with Topic Questions

Participants were invited to choose a breakout session on one of three topics: (1) Studying Kidney Developmental Disorders from Gestation through Childhood, (2) Translating WGS to Hypotheses and Model Systems, or (3) Environmental Exposures and Nutrition.

Report Back and Discussion

Group 1: Studying Kidney Developmental Disorders from Gestation through Childhood

Group 1 discussed how to create a continuum of research that spans intrauterine development through birth and childhood, noting that the incidence of chronic kidney disease in existing cohorts is a challenge. Ancillary R01s may be a good mechanism for using existing data. Many opportunities to collaborate across disciplines exist—many centers have maternal fetal research units, and a neonatal kidney collaborative may provide some opportunities. This work may intersect with research outside the United States.

One of the group's most extensive discussions was on barriers to collaboration, such as ways to ensure that all NIH-sponsored data are findable and can be merged with nephron interests. The group also discussed whether the widespread use of prenatal ultrasound can be used to systematically study congenital kidney disease and, if so, how such a cohort could be identified and followed. Fetal anomalies are not identifiable until the 20-week ultrasound, so complementary cohorts could be developed for deep phenotyping in follow-up and more shallow phenotyping of a larger group. PEDSnet could be used to identify cohorts of at-risk fetuses. The group also discussed how to recognize and improve the burdens placed on families to participate in research, as well as how to harness a desire to participate to improve the field.

Needed phenotyping strategies include identification of an animal model and consensus on how to ethically approach the issue from a tissue perspective. Collaborations will be required to discern and follow anomalies that are coincident with congenital kidney disease.

Group 2: Translating WGS to Hypotheses and Model Systems

Group 2 pointed out that WGS is expected to find SNPs in many untranslated regions, but intronic regions are not conserved, creating a large number of variants that are hard to interpret. Short reads have blind spots and require knowledge of which promotors, repressors, and enhancers to target. A large number of normal cases is needed to compare probands to, and creating model systems is difficult before WGS is performed and regulatory variants are identified.

Computational tools are needed to understand WGS heterogeneity and combinations of SNPs, as well as translate that information to testable hypotheses. Long reads can fill gaps, but backbones must be established to integrate data. Epigenetics can provide broad markers to determine whether a gene is

activated. Larger pathways can be narrowed using specific genes, drugs, or CRISPR. Group 2 also discussed the need for phenotype data and data on somatic mutations and mosaicism.

Group 2 discussed the need for collaboration with large data sets or creation of a consortium to interpret and integrate data adequately. Although NIH is working on such efforts, more collaborations similar to those featured in this workshop are needed to collect samples prospectively in a way that allows them to be used with new technology. A consortium could allow specific sites to perform specific work. For example, the Epi4K Consortium was able to target specific centers depending on the expertise needed to review the data.

Group 2 also discussed which models are appropriate for validating genetics. The field has many options available, but it is limited by lack of a platform to better connect patient genetics with experts in ideal model systems, as well as the need for normal developmental data for comparison. Prenatal sequencing raises specific concerns for patients and caregivers, such as effects on future treatment and qualification for studies. Obtaining autopsy samples is associated with data privacy and sharing concerns, and consent is critical.

Group 3: Environmental Exposures and Nutrition

Group 3 discussed how to detect environmental exposures and potential effects on kidney development and nephron endowment. Multiple biomarkers are needed, and researchers must differentiate between host response and longer-term effects, as well as consider parental exposure. NICU conditions and medications should be recorded, and various samples can be collected to measure exposures. Biomarkers are needed for tubular function and differentiation between structural and functional kidney injury. The group also discussed gathering census-level or geolocated data, as well as information about water sources, to determine local exposure risks.

Group 3 discussed how to leverage existing resources and studies, such as ECHO. Amniotic fluid may provide information on exposure, but the ability to estimate kidney function or long-term clinical health is limited. Kidney development and nephron endowment information has poorly defined clinical significance, especially given the heterogeneous nature of congenital kidney disease.

To discern the effects of nutritional deficiencies and excesses on kidney development, nutritional epidemiologists are applying serial questionnaires, but model systems are needed to define mechanisms and clinically meaningful outcomes with correlations to human data. The group also discussed the role of the placenta in controlling the supply of oxygen, nutrients, and toxicants to the fetus and whether pregnancy status limits potential novel interventions. Group 3 also discussed what developmental periods might be affected by nutrition and environmental exposures, assessment of breast milk versus formula regarding effects on kidney development, and whether exposure nutrition studies should be broad or local. Environmental exposure effects on CAKUT remain to be studied, and addressing disparities in access is important.

Group 3 also discussed the potential role of kidney biopsies in these studies and the need to correlate tissue findings with mechanistic pathways of disease, noting that the NIDDK repository is a potential source for biopsy data. The group recognized the role that families play in clinical care and research and the need for report-back options during consent.

Discussion Highlight

- Participants agreed that this is an exciting time for kidney research, and engaging the next generation of clinicians and researchers is important. Dr. Star commented that the best way to

stimulate a field is to show outsiders that many questions, tools, and data are available and the questions are feasible to answer. He noted the importance of expanding the group of people interested in patients with congenital kidney disease.

Closing Remarks and Discussion

Dr. Star reiterated the importance of bringing together people from different communities and thanked the speakers, audience, and organizers. He summarized that each group of participants has different interests, but finding common ground is critical, and this workshop allowed the parties to listen to one another and to understand and translate between different terms. Basic needs for the field include a standard lexicon, better phenotyping, mechanisms of post-birth kidney development, and understanding of arcading and LBN. CAKUT is a complex disease with many unknown causes, and existing cohorts are small, so a large cohort study is needed to identify genetic, environmental, and nutritional causes and develop a data atlas that allows in-depth analysis. A better understanding of acute kidney injury in neonates also is needed.

A few simple first steps emerged, such as including patients and families at every level, creating a standard lexicon, validating measures of nephron number, defining priority populations, leveraging existing cohort studies, defining phenotyping and genotyping strategies, screening loci and studying the mechanisms of genetic disease, and linking stressors to epigenetics to create multivariate scores for prognosis, therapeutic decisions, and outcomes. Potential pathways forward include the Kidney, Urology, and Hematology Innovative Science Accelerator, the R21 in Catalytic Tool and Technology Development in Kidney, Urologic, and Hematologic Disease, and a program in High-Impact Interdisciplinary Science in NIDDK Research Areas. Dr. Star encouraged potential applicants to contact the NIDDK to discuss their ideas.

Adjournment

Dr. Mendley thanked the participants and noted that her team will consider the best way to move forward after this workshop.