National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)

URINOLOGY THINK TANK

Two Democracy Boulevard
Room 701
Bethesda, MD
Executive Summary

February 9, 2015

Draft Meeting Summary

MEETING OPENING

Welcome

Robert Star, M.D., Director, Division of Kidney, Urologic, and Hematologic Diseases (KUH), NIDDK, NIH, Bethesda, MD

Dr. Robert Star welcomed the meeting participants and explained that the meeting's purpose was to test a new concept that urine is not just a liquid that contains various constituents that might inform about the body's condition. Rather, there is a key question: Could these various constituents in urine be biologically active? In the Middle Ages, urine was viewed as an indicator of systemic health. Urine could be looked at, smelled, and even tasted to determine the internal state of the body. Homer Smith and R.F. Pitts developed urine markers to figure out how the kidney functions. But no one had formally discussed—until the Urinology Think Tank meeting—whether active constituents in urine contribute to bladder function and homeostasis. The purpose of the meeting is to explore the evidence that urine constituents play a role in bladder health or dysfunction. Does the idea of biological communication from urine constituents make sense? Is it worth pursing and, if so, what should be done to move forward? Dr. Star acknowledged the efforts of Drs. Tamara Bavendam and Deborah Hoshizaki in organizing the meeting. Dr. Bavendam thanked the participants for attending the meeting, which she emphasized would be informal. She asked the attendees to introduce themselves and describe why they chose to attend the meeting. The participants' introductions revealed that they comprised a spectrum of disciplines, such as comparative evolutionary physiology, basic science, nephrology, kidney and urologic medicine, pediatrics, metabolomics, and proteomics.

Why Are We Here?

Tamara Bavendam, M.D., M.S., NIDDK, NIH, Bethesda, MD

Dr. Bavendam described the evolution of her interest in the idea that urine might be more than simply a waste product. When she began her practice as a urologist in 1987, her office was filled quickly with women who had received all of the procedures, treatments, and surgeries available at that time without resolving their illness; they were seeking a different approach. As the women described their experiences, themes emerged that led Dr. Bavendam to think about urologic conditions differently.

One consistent theme was the variability of the patients' symptoms. Although the women reported always having to urinate or experiencing pain, closer examination revealed times of the day when symptoms were less severe. The most symptomatic women had very concentrated urine; it made sense that having a particular solute concentrated in the bladder might be more irritating. Dr. Bavendam learned that her patients felt better and their conditions could be managed through urine dilution, an insight of tremendous value over her 20 years of practice.

Urologic conditions are difficult to study in women. Urine contains a high solute concentration, raising the question of whether that could affect the urothelial dynamics. Given that urine is constantly in flux and the need to maintain solute and water balance in the body, it made sense that perhaps changes in the urine were influencing symptoms.

During the years of her urology practice, Dr. Bavendam could not explain why ingesting certain foods triggered bladder infections, as foods consumed are not excreted in urine. Rather, foods are processed in the gut and studied through fecology, a term that inspired the label urinology. That diet affects symptoms has been observed by many and is well known. Because it is difficult to study in a controlled environment what women are ingesting and what is being excreted in their urine, Dr. Bavendam's thinking turned to rodent studies as a way of determining if diet could change voiding behaviors and if yes, how? In controlled environments, scientists can collect evidence that changes in the diet affect urine and can begin to understand the effects. Thus, mechanistic animal studies would be an approach to answering some of the questions based on human observations.

The idea for the Urinology Think Tank meeting was conceived during several conversations within KUH. At one point, Dr. Hoshizaki asked Dr. Bavendam if urine could be biologically active and introduced the perspective that urine might be responsible for homeostasis and health. It is known that organs communicate through innervation, but another crosstalk mechanism might involve specific kidney secretions in the urine that reflect whole organism health and provide signals for interpretation by the bladder. These discussions led to the conceptualization of the Urinology Think Tank meeting, aimed at convening diverse experts to help clarify the issue. The potential biologic activity of urine is not well represented in the scientific literature, although it is known that neutrophil gelatinase-associated lipocalin (NGAL) is secreted in urine and has some effect on the expression of uropathogens. At a meeting in 2014, researchers presented data on the metabolomic profile of urine and its variations in patients with different clinical diagnoses. These results provided hints that the idea of urine being biologically active was worth further consideration.

Dr. Bavendam's primary research focus is prevention. From that perspective, she asked if having frequently changing liquid that is constantly in the bladder plays a role in the health of the bladder and homeostasis. It is known that tobacco toxins are excreted in urine, contributing to bladder cancer, and there already exists accepted science concerning urine constituents that affect bladder health. With the advent of microbiomics, metabolomics, and proteomics, tools now are available to answer questions that scientists in the past could only answer with their tools of sight, taste, and smell to make judgments about health.

Until recently, students were taught that the urine is sterile, but now scientists know that is not the case. The bladder might be similar to the bowels and the vagina in containing both "good" and "bad" bacteria that can mediate health or cause symptoms. It is not surprising that women experience more frequent bladder infections given their anatomy. On the contrary, it is surprising to find women who have never experienced a bladder infection, raising the question of why researchers were not trying to understand what differences in those women's bodies and urine might explain why they do not experience infections.

Dr. Bavendam explained that NIDDK is responsible for research on benign urologic conditions, including those of the bladder. The study is challenging because an ailing bladder is known by its symptoms, but the symptoms are not specific. The same symptoms could be related to many different underlying pathologies. In the past, there were three categories of urologic diagnoses: prostate conditions in men, interstitial cystitis (IC), and urinary incontinence (UI). Although treatment studies generated high-quality information, they also raised many questions; for example, comparative trials did not show that one treatment was better than another. That led physicians to ask if enough was understood about clinical diagnoses to select better treatments, inspiring the Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) network approach to the study of chronic pelvic pain study, which has focused on IC and chronic prostatitis. MAPP is taking a nongendered approach to thinking about urologic issues and is expanding the frame of reference to include the bladder, as well as other organs.

The Symptoms of Lower Urinary Tract Dysfunction Research Network (LURN) is organized around nonpain urinary symptoms and is taking a similar approach to MAPP, exploring symptomatic conditions at a basic level and examining how symptoms are measured. Although patients tend not to complain about urinating infrequently or lacking sensation in their bladder—because they do not mind needing to urinate less frequently—not experiencing normal bladder sensation may be important to identifying specific phenotypes. Current lower urinary tract symptom (LUTS) diagnostic tools pose no questions about how a patient perceives his or her bladder. Questions address urinary urgency or pain, but do not ask about the presence or absence of a filling sensation. Both MAPP and LURN are collecting urine and serum specimens from well-characterized patient populations, creating many opportunities for discovery about symptoms. How such research might be incorporated into bladder health is yet to be determined.

A third network, the Prevention of Lower Urinary Tract Symptoms Network in Women (PLUS), is being developed to study women before their symptoms arise—or when they are mild—with a goal of working toward primary or secondary prevention. The more that is learned about these conditions, the more complex they become and the harder the challenges appear in identifying the keys to solving the problems and minimizing their impact on women. One goal is to determine what actions can prevent the problems so they never develop to the point that only limited treatment options are available. Accomplishing this objective will involve examining all elements that may be related to bladder health, including urine. A better understanding is needed about what can be manipulated to maintain bladder health over a lifetime.

Dr. Bavendam thanked the participants for contributing to the intellectual exercise of the Think Tank, which could produce enough information to prompt a larger meeting in the future. First, however, the field of knowledge and interest concerning urinology must be garnered.

Discussion

Dr. Roger Dmochowski stated that he was interested in the possible role of urine in allergy and immunological responses. For example, a half-dozen women with previously undiagnosed gluten neuropathy managed their diets after diagnoses and their urinary symptoms resolved. He asked if that experience might be representative of a metabolic byproduct of neuropathy or a relationship between the gut

and bladder. Urinary biologist Dr. Michelle Southard-Smith has examined the migration of crest cells comprising the lower urinary tract (LUT) and hindgut and traced them back to the same place. Research must consider the afferent function relationship with bladder function. The function is immunologically reactive in the gut and might be the same in the bladder. It is a fascinating aspect of that modulation and could provide insights to those who do IC signal bladder abnormality work.

Dr. Jonathan Barasch asked if the diet that Dr. Bavendam mentioned referred to fluid intake only. Dr. Bavendam responded that in her experience, it was not just fluid intake. Literature supports findings that caffeine increases bladder muscle activity. Carbonation or spicy and acidic flavors—a standard set of irritants, especially in the IC population—also will trigger symptoms. Even in the non-IC population, patients report substances in their diets that trigger bladder symptoms.

Dr. Joe Williams asked about connections between food and bacterial infection. Dr. Dmochowski responded that the issue pertained more to the modulation of afferent behavior. The problem is that women are habitually trained regarding any urinary symptoms. Patients might contribute to exacerbations of the urgency and frequency of pain because they have been habituated to say they have symptoms and therefore have an infection, and the Pavlovian response of the clinician is to prescribe antibiotics. The other issue is how the microbiome has been altered by the overuse of antibiotics. Dr. Williams added that there could be an evolutionary component to the response.

Dr. Tony Buffington noted that there have never been blind control trials of any nutrient ingredient or chemical ingested by mouth and its effect on the bladder, to differentiate between what is consumed in the food and determine whether a particular ingredient (e.g., a potential irritant, such as capsaicin) makes it to the bladder whole or is metabolized.

Dr. Patrick Seed noted that the world of complex microbiota could be transforming the urine constituents. Even what is measured in urine metabolites might not be providing biologically active minor constituents because there are a mucosa-associated organisms in a gradient; the organisms potentially could be producing a critical signal right next to receptors for those metabolites. Very little is understood about how urine constituents are being changed in the biota of “normal” individuals. Much remains to be learned.

Dr. Stephen Hewitt added that an additional challenge facing investigators is that historically urine has been considered from a chemistry perspective. Starting in the 1850s, physicians had tools to examine urine: color, odor, specific gravity, taste. The chemical analysis of urine was quickly adopted by the 1860s. Although urine could be microscopically evaluated, it always had to be examined visually. As result, an enormous bias occurred in which modern medicine analyzed urine chemistry and has not considered urine biology. Because urine is not treated as a biological specimen, doctors are handicapped in finding answers to their questions. Work is needed to develop the right tools. For example, clinicians routinely request a 24-hour urine sample. If the test does not account for what a patient ingests in fluid and food, nothing will be accomplished. Requesting 4-hour aliquots would provide a better understanding of the physiologic hormone cycle of the urine throughout day. That single incremental improvement would provide much more information.

Dr. Warren Hill asked if any clinical trials had been conducted on the effects of urine dilution, which must be observed by urologists everywhere and is worth investigating more rigorously. Dr. Bavendam responded that she had tried to plan numerous trials, but collecting so much data is very labor intensive and funding has not been available.

Dr. Paul Kimmel described how his mother had suffered from bladder dysfunction related to multiple sclerosis for 60 years. Her response was to dehydrate herself, taking one cup of coffee in the morning. That type of common behavioral response might create a predisposition to having extraordinarily concentrated urine.

Dr. Hewitt noted that fecal transplants are now used and asked about the possibility of urine transplants.

SPEAKER PRESENTATIONS
Moderator: Deborah Hoshizaki, Ph.D., NIDDK, NIH, Bethesda, MD

Normal Mechanisms
Jonathan Barasch, Ph.D., Columbia University, New York, NY

Dr. Barasch described the Iron Problem, noting that the kidney has two main functions, absorption and secretion. The molecules that it spends a lot time reabsorbing are filtered proteins and other substances, one of which is iron. There is little iron in the urine; calculations indicate that more than 1 milligram (mg) per day is reabsorbed by the kidney. An adult body requires 1–2 mg/day, so the iron must be reabsorbed for different reasons. The body produces ferrous iron, but it is well chelated. The more common form of iron is toxic, reacting with oxygen to generate active species, and must be removed. This form is highly insoluble, forming polymers in water and precipitating with phosphates.

Many iron transport proteins are found in the kidney and intestine. A number of receptors and processes are understood, but many molecules remain to be studied and understood. Kidney-specific gene knockouts are needed to determine how iron is removed so that it

does not enter the urine. Some iron forms are bound to transferrin, and others are not. The kidney has a variety of defenses against moving iron into the urine, which is part of what Dr. Barasch studies. At birth, the apical surface of the kidney contains the transferrin receptor, but later in life the receptors are located distally. Collecting ducts also have transferrin receptors, but with development they are lost from the proximal tubules. When the transferrin receptor was knocked out in experiments, iron collected downstream, causing cystic changes in the kidney and epithelia filled with ferritin. The results demonstrated that the upstream transferrin receptor is important for removing iron from the urine. The kidney must capture and remove iron-transferring proteins, such as the transferrin receptor TFR1.

NGAL is a small molecule that, when injected into the blood, is taken up by proximal tubules and shifted to lysosomes. It can carry iron under certain circumstances. The kidney prevents NGAL from passing into the filtrate through the protein megalin. When megalin is knocked out, NGAL is found in the urine. Otherwise, when NGAL is injected experimentally, very little ends up in the urine, and most of it accumulates in the kidney. Because NGAL knockout animals became very sick, Dr. Barasch's team created hundreds of mutations of NGAL surface residues. Eventually they found a mutation of NGAL that directly enters urine, bypassing megalin in the proximal tubule and carrying iron into the urine. The results showed that the proximal tubule contains megalin and transferrin receptors early on, and other mechanisms contribute to removing iron from the filtrant. In acute kidney injury (AKI), involving damage to the nephron, a 10-fold increase in urine iron is observed that may worsen the AKI, as chelators will improve the condition. Classic studies suggest that the movement of iron into the urine can be potentially toxic. Although the kidney has a variety of mechanisms to remove iron from the urinary space, it is believed that those mechanisms are damaged in both acute and chronic diseases.

NGAL is taken up by the proximal tubule, and it is observed accumulating intensely in the urine in damaged kidneys. It appears quickly before creatinine levels rise, as quickly as 2–3 hours after a surgical procedure. The more severe the damage, the higher the NGAL levels. A study showed that a single drop of urine in the emergency room can be used to create highly predictive assessments for the week ahead, with patients having the most damaged kidneys either undergoing dialysis or dying within 7 days of the emergency visit. To study what is happening in the kidney that generates so much NGAL, a luminescent mouse was created. The amount of NGAL was shown to depend on ischemia. When food and water were removed from the mouse, there was no NGAL, even with rising creatinine. The same phenomenon is observed in patients. Furthermore, inflammatory, ischemic, or toxic tissue injury is required to make NGAL. Other experiments showed that NGAL is a critical antimicrobial, as knocking out a specific immune system-related transcription factor (toll-like receptor 4, or TLR4) in intercalating cells markedly reduced NGAL in urine. Moreover, adding NGAL to bacteria slows colony growth for many hours.

Although studies have provided significant knowledge about NGAL, many questions remain: If NGAL is a narrow spectrum antibacterial, are there other ligands? Does NGAL have an endogenous ligand? Why is NGAL expressed so quickly? Is NGAL unique in its specificity for ischemic damage? Do alpha intercalated cells express other antimicrobial proteins? To further study some of these questions, a new mouse model was developed to enable the extraction of RNA from any cell in the body without dissection. A disease map built from a dissected kidney will soon be available online describing the expression of RNA in specific cell types in response to ischemia and other conditions.

In conclusion, Dr. Barasch summarized that the kidney prevents iron and iron-carrying plasma proteins from entering the urine. The barriers include megalin and the transferrin receptor. The kidney epithelia upregulates NGAL upon damage, but not in response to volume depletion. NGAL is rapidly secreted from the thick ascending limb of Henle's loop and intercalating cells, quickly reaching the urine. NGAL is a narrow spectrum bacteriostatic protein and may chelate iron by other cofactors. In response to bacteria, intercalating cells secrete NGAL and protons, indicating that urine defense is carried out in part by the kidney. Dr. Barasch added that the topic of the Urinology Think Tank meeting would be a wonderful focus for future research.

Discussion

Dr. Jayoung Kim asked about protein expression in response to kidney damage. Dr. Barasch clarified that 10 years ago, epidemiologists combined several conditions under the term AKI. The term includes a 12 hour rise in creatinine. A transient increase in creatinine does not generate damage, but if it is prolonged the results are ischemia, hypoxia, inflammatory diseases, infection, obstructive diseases, and others.

In response to a question, Dr. Barasch noted that a scientist at Columbia University scanned hospital records for New York Presbyterian Hospital, collecting hundreds of thousands of data points. Using an algorithm on baseline creatinine measurements, the researcher was able to query how long a creatinine rise lasts. The results were divided into rapid resolving (within 48 hours) and prolonged (greater than 5 days). For the middle zone, no conclusions could be drawn. The researcher found that 70 percent of the people entering New York Presbyterian Hospital had prerenal azotemia or transient azotemia, rising and falling within 48 hours. If the condition lasted for longer than 5 days, the chances of recovery were reduced linearly with age. Volume depletion was present in the elderly.

Dr. Hoshizaki asked about the correlation of NGAL induction with kidney injury or bacterial infection. Specifically, she asked if bacterial

infection in the bladder produced the induction of NGAL in the kidney. Dr. Barasch said that bacteria in the bladder of C57 mice do not reflux to the kidney. When 25−50 microliters (mL) of bacteria were introduced into the mice, the luminescent bacteria were not visualized in the kidney. Furthermore, when the kidney was removed for evaluation, bacteria were not cultured from the organ. Dr. Barasch noted that the refluxing mice are TLR4 dependent, but more research on the issue is needed. In the presence of cystitis, NGAL, interleukin-1 (IL-1), and other proteins are expressed in the kidney despite the inability to culture bacteria[?in the kidney?]. Since that system is significantly TLR4 dependent, it is necessary to conclude that some reflux is occurring. Dr. Barasch noted another experiment he conducted involving enterochelin, which can induce NGAL expression when injected into a mouse. That result is not TLR4 dependent. Enterochelin is a very small, water-soluble molecule that could be refluxing. It is unclear why cystitis is activated.

Dr. Star asked for clarification about the antibiotic experiment. Dr. Barasch responded that when bacteria are injected into the bladder, IL-1 , IL-1 , and so on are produced. In the same model, the kidney makes IL-1 , IL-1 , and other circulating cytokines. These cytokines could be identified from the blood or small amounts of reflux. It is uncertain, but Dr. Barasch believes that small amounts of reflux occur through TLR4.

Dr. Hewitt stated that it sounded as if the intercalating cells acted as a bidirectional sensor in the system, with reflux occurring in response to the TLR4 system. If reflux is coming from some other system, it is sensing the same way downstream. Drs. Hewitt and Barasch discussed the role of the intercalating cell in the process. Intercalating cells secrete protons into the urine and could provide an antimicrobial function. How they sense TLR4 and respond so quickly are among the many questions.

Dr. Kimmel noted that in the past, Dr. Barasch considered NGAL as a potentially reparative molecule in the kidney. He asked if Dr. Barasch had examined patients with hemolytic anemias who contain large amounts of iron in their urine, which has always been considered a mechanism for kidney injury. He also asked if Dr. Barasch had any information regarding interactions with hypoxia-inducing factors. Dr. Barasch said that minor hemolytic damage was not sufficient to raise creatinine in many patients despite an inflammatory response. Creatinine increase certainly occurs with rhabdomyolysis, which produces enormous NGAL expression. Research shows that hypoxia-inducible factors (HIFs) can drive NGAL, but at a small level of two- to fourfold. The real factor is NF-kappaB, which activates polymerase II progression. Regarding its reparative action, several publications suggest that adding NGAL can reduce damage. For many years, it has been thought that using bacterial siderophores can reduce damage, such as in the case of heart disease. Although that field was abandoned many years ago, it may be worth resuscitating.

Dr. Hoshizaki asked Dr. Barasch's view on whether intercalating cells can respond to bladder signals. He responded that the response occurs in cystitis models, but the mechanism is unknown.

Water Balance
Joe Williams, Ph.D., Ohio State University, Columbus, OH

Dr. Williams stated that his presentation would provide an overview of water balance in humans and animals. From his perspective as an evolutionary physiologist, the creation of homogenous rat and mice models by medical researchers has resulted in highly artificial systems that provide little information about evolutionary biology. Having been in his field for a long time, however, Dr. Williams said that he was heartened by the establishment of evolutionary medicine as a new field at Michigan State. This new field addresses questions such as why, when a person has a fever, the immediate clinical response is to reduce it when fever is actually an evolutionary response to bacterial infection.

As a brief reminder about water balance, Dr. Williams noted that humans make approximately 300 mL of metabolic water per day and obtain 2,200 mL/day from food and drink, more than most other animals. This results in the production of a more dilute urine than other animals. In feces, humans lose approximately 100 mL/day and from urine about 1,500 mL/day, also more than most animals. Dr. Williams' work has focused on evaporative water loss, including in animals living in the desert. He has partitioned this process into respiratory and cutaneous water loss and has focused considerable work on how the skin modulates water loss.

Broadly speaking, water influx must equal water efflux. In humans, there is a water balance of about 2,500 mL/day, with 900 mL/day of evaporation loss. The kangaroo rat, in contrast, derives most of its water from metabolism and loses about 0.5 mL/day, mostly through evaporation. Dr. Williams has studied the evolutionary biology of water loss through the skin, focusing on larks and birds in Saudi Arabia, Spain, and the Netherlands. Cutaneous water loss comprises a significant part of water balance in most animals and in humans drinking normal amounts of water; birds, for example, lose approximately 60 percent of their water through their skin.

Dr. Williams studies skin lipids using mass spectrometry. Ceramides, free fatty acids, and triglycerides are the normal lipid constituents in human skin, which can moderate water loss through skin. Especially interesting from an evolutionary perspective are cerebroside molecules, which are not found in human skin except in cases of Gaucher's disease. Birds have cerebrosides in their skin, comprising as much as 40–50 percent, even more in desert areas with markedly less water loss.

In contrast to humans, who cannot survive a 12-percent water loss, camels can lose 25 percent of their body mass in water before physiological complications occur. Camels can live for 7.1 days without water—their owners release them into the desert knowing they will return to drink. In Saudi Arabia, Dr. Williams studied the physiology of the Arabian oryx. The oryx is designed to live in the desert and never drink, obtaining approximately 350 mL water/day in food in an environment where the ambient air temperature hovers around 44–45 C. The oryx is a remarkable animal that produces very concentrated urine and extracts a large amount of water from its feces, which are a powder.

Animals eliminate nitrogenous wastes in various ways. The mammalian kidney possesses an antidiuretic hormone that collects water. Lizards have a bladder that often is used as a canteen; during dry periods, water is absorbed out of the bladder. Birds in Arizona deserts have a remarkable ability to concentrate urine. Many birds reflux urine into their lower digestive tract where water is further extracted. Dr. Williams noted that the urine and plasma osmotic ratio of birds and other animals cannot be compared, as the mechanisms are quite different from an evolutionary perspective.

Discussion

Dr. Barasch asked if bladders reabsorb water. Dr. Williams noted that the bladder does not reabsorb water in most mammals, but research on bladders and urine has been limited because of scarce funding. Dormant bears recycle many bodily constituents, though they are not truly hibernating like animals on the Arctic tundra. Dr. Hill noted that aquaporins are found on the bladder in the basolateral membranes, so presumably a mechanism exists there for water to passively diffuse across the membrane where water absorption might occur. Dr. Buffington asked how much genetic variability occurs in partitioning water loss. Dr. Williams responded that much genetic variability exists, even within the same species. Given the complexity, evolutionary biologists take an average within the species before making comparisons. He acknowledged that there might be variability in the constituents of human urine between individuals.

Dr. Kimmel asked if animals with high urinary osmolality experience bladder damage; perhaps humans are outliving their protective mechanisms? Dr. Williams replied that the oryx, which lives approximately 20 years, does not experience damage. Dr. Star asked about the bladder acting as a canteen for water and whether the signal was vasopressin or some other signal. In his response, he cited a study by Dr. David Black of the University of Arizona in which the researcher purposefully removed the urine from lizards, along with a control group, and studied the survival rate after the lizards were released. The survival rate for the controls was much higher.

Dr. Hewitt commented that the transitional epithelium of the bladder, which is complex, exists for an evolutionary reason. A newer study examines the bladder's FTFR3 receptor, which is associated with bladder cancer. It is an example of an evolutionary polymorphism affecting function. Dr. Hewitt commented that critical pathways can be identified by leveraging knowledge about all organisms and becoming less anthropocentric. Dr. Star agreed and noted that at a recent microbiome meeting, researchers made the argument that individual signaling molecules might function in different pathways conserved across species. Mice and men might use different forms of interleukins, although the cassette is the same. The challenge is to learn the correct mechanisms from the animals.

Dr. Hoshizaki asked if, for animals with concentrated urine, any researchers are studying the structural differences in the urothelium. Dr. Williams responded that very few researchers study bladders in an evolutionary context.

Bladder Microflora
Patrick Seed, M.D., Ph.D., Duke University, Durham, NC

Dr. Seed noted the explosion of interest in the microbiome and described what is known about the urinary microbiota. He presented a series of questions: Is there an endogenous urinary microbiome? If so, are there core urinary organisms? Are the urinary microbiota developmentally dynamic? When does it start? He would argue that humans are not born sterile, so the urinary microbiome may exist in utero. Are there rules of succession? Is it sex specific? Is it communicable? Are there family cohorts, as with families' common constituent gut biota? In examining evolutionary coadaptation, it will be incredibly important to understand what other organisms are present. Schistosomiasis and its dramatic effect on the bladder and urinary tract are well known, and there may be many other examples.

If one accepts that there is an endogenous microbiota, why would it matter? Are shifts in communities associated with specific diseases? Are there microbiomes that are commensal or—more profoundly—symbiotic, and thus giving back to human physiology? How does the bladder and its microbiota serve as the genesis of urinary tract and non-urinary tract diseases? Focus has been on the gut and its role in everything from type 2 diabetes to neurologic disorders, but how many diseases arise from dysbiosis in the urinary tract? The LUT is thought of as an immunoprivileged site; yet deep studies of the antigen response to bladder infection reveal a profoundly complex situation. The LUT is not an immune-tolerant organ. How LUT responses ignite autoimmunity though mimicry is a key question. If there is a succession in the development of a microbiome, how does that program the expressed genome and produce lifelong function?

Microbes are well known as physical barriers, such as between the epithelium and urine. They function as immune modulators and are excellent bioreactors for nutrients, xenobiotics, and bioactive metabolites in a two-way interaction. Gut microbiota are known to activate

the intestinal tract at a local level, including local metabolism in which enterocytes are fed fatty acids to provide energy, and local neural activity produces gut motility. More distal effects also are apparent. In hematopoiesis, if certain components of enteric microbiota are missing, granulocytes do not work as well. Metabolic and central nervous system (CNS) effects also exist—shown in studies of the gut/brain axis—with the intestinal biome as part of the dynamic. All of the findings about the intestines can be applied to the bladder.

A large number of small molecules are made primarily by the microbe or are biotransformations of xenobiotics. Additionally, many immune constituents in the gut are present and active in the bladder. Commensal microbiota with immune suppressive and active agents are well understood. From a developmental standpoint, when the microbes of normal colonized animals are transplanted into the gut of germ-free animals lacking experience with microbes at 1 week or 3 weeks, it provokes a differing response. Delay in colonization produces a huge effect on bladder and gut function, as seen with women and children who receive antibiotics when they are born: immunologic defects can be retained well into adulthood. A more provocative idea is scientific evidence showing that the gut microbiome, depending on the composition, likely produces different responses to an inactivated flu vaccine.

Dr. Seed described the benefit of probiotics in the gut-brain axis. Citing a Proceedings of the National Academy of Sciences publication as early evidence, he highlighted work in in which feeding the probiotic Lactobacillus to mice produced decreased anxiety and better tolerance of forced swim tests. The research demonstrated connections between the gut microbiome and the vagus nerve; there is no reason to think that the urinary tract microbiome could not also participate in this process.

Dr. Seed referred to the dogma that the urinary tract is sterile. He presented a recent collection of studies examining the urinary microbiome and demonstrating great diversity. Many early studies were related to sexually transmitted diseases and thus were measuring urethral organisms. More recent studies have focused on microbes present in the urine of principally normal, healthy individuals.

A key question concerns how the microbiome is measured. Approximately 80 or 90 percent of organisms associated with humans are not readily cultivatable. The classic method to identify species involves sequencing the 16S ribosomal marker, a taxonomic marker at the genetic level that is highly conserved but with sufficient variability to examine species-level associations. Data from a relatively small study involving healthy individuals across a wide range of ages in the United Kingdom produced several key findings. Phyla-level data on midstream urine samples showed that most of the microbiome species fell into one large phylogenetic group, or urotype. Amid the diversity, all individuals' samples were dominated by one phyla, Firmicutes.

Data on urinary microbiome by genus show more diversity, although some individuals are still dominated by certain organisms. Many of the organisms also are found in the gut and vagina, while others are not. Many urine microbiota that are not routinely cultivatable occur at levels of high abundance, which is important when considering the concept of biologically active urine. If a large percentage of the microbial species in urine are viable, they could be performing important functions. Questions of interest concern organisms that are seen across all ages versus those that are gained and lost over time.

Dr. Seed conducted an imputed analysis of the United Kingdom study to understand the potential of the genomes in terms of metabolism and physiology, using reference genomes to create regulatory maps. He developed a non-metric multidimensional scaling plot to focus on the variables that most likely show differences in populations. The analysis suggested that many different species could be filling the same niche, or the significant diversity that is observed could encode different physiologies.

The study by Drs. Linda Brubaker's and Alan Wolfe's group at Loyola University examined constituents present in the urine of a control group and the urge urinary incontinence (UUI) group and found a number of organisms highly associated with UUI that were not present in the control groups. The study must be replicated, but it contributes to work on the hypothesis that there exists a community with different urinary organisms in those with UUI. The results lead to mechanistic studies that ask how different organisms contribute to disease. It is likely that there are dramatic differences in the genomes of organisms that dominate.

Dr. Seed examined data for a series of individuals with spina bifida, including clean intermittent catheterization (CIT) and volitional voider groups. All patients possessed similar bladders and urodynamics. It was expected that the CIT group would have different microbiota than the volitional voider group, but this was not the case. The capacity for the urinary bladder to have complex microbiota and for individuals to be non-symptomatic is substantial. Individuals in the study with infrequent UTIs tolerated a hugely diverse microbiota, presenting an opportunity to learn what leads to such differences in microbiota.

Dr. Seed described his research studying canonical and noncanonical pathways of inflammation and metabolic signaling. A study on asymptomatic bacterial strains found that phosphorylation of a particular protein reduces inflammatory pathways as part of the unique commensal relationship developed by asymptomatic bacteria over time. Other studies have examined the differential risk for UTIs to determine whether polymorphisms prevent inflammatory signaling or shift the microbiome to a more protective state.

How microorganisms participate in the bioreactive metabolism of the urinary metabolites is completely unknown. It is clearly known that UTIs exacerbate dementia, demonstrating the bladder/brain connection. Another publication examines very specific gut bacteria that

drive autism. The findings raise numerous questions. The prospectus is that research must understand the array of microbiomes among healthy individuals, across the lifespan, for all ethnicities and race. Scientists must endeavor to understand the interaction of microbiomes with development of the urinary tract, the persistence of the urinary tract phenotype, and urinary metabolite-microbiome relationships. Cross-transplantation studies are needed because much can learned when biota are transferred between animals. Cancer and pre-cancer microbiomes in the gut and liver should be explored. Finally, the big question is whether a bladder/brain connection exists whose communication the microbiome helps to facilitate.

Discussion

In response to a question from Dr. Hoshizaki, Dr. Seed responded that many researchers study E. coli found in urine and, in his view, those E. coli are the "losers" because they have not figured out how to attach to and invade an epithelium, often to subvert the immune system. Researchers have used feces to study mucosal-associated bacteria. The tendency is for feces to contain an average of the constituents that pass through the intestinal tract. These bacteria are in many cases functionally different from those found in the mucosa, however.

Dr. Williams asked about the source of urine bacteria. Dr. Seed responded that the canonical answer is that the bacteria are all ascending. Women have significant flux between the perineal, vaginal, and gut microbes. New organisms frequently fluctuate into the urine. In males, it differs. In the first 6 months of life, boys have a much higher likelihood of UTI than girls, an unexplained phenomenon. If blood samples were taken from all of the meeting participants, bacterial genetic signatures would be frequently found as evidence of the continuous trafficking of viable and phagocytosed bacteria through the body. For breastfeeding women, 100 percent would have bacterial sequences in their blood, compared with ~20 percent of those in the general population.

Dr. Kimmel asked if the diagnosis of UTI was made by symptoms, and if those with neurogenic bladders might not have symptoms. He asked if there might be overlap between those with infrequent and frequent infections. Dr. Seed stated that there might be overlap. Various groups have studied intracellular invasion and replication of bacteria within the apical epithelium bladder. Studies have been E. coli-centric, but there could be a very large reservoir of organisms present. It is known that intracellular pathogens have very different ways of subverting immunity. It is a challenge that will require animal models studies for better understanding. Phage, viral, parasitic, and other organisms also may be part of the whole process.

Dr. Barasch asked if the mouse microbiome is likely to be the same as that of human, or if a humanized mouse can be created. Dr. Seed responded affirmatively that creating humanized mouse is possible, noting that Dr. Jeff Gordon's group pioneered such work using standard gnotobiotic technology. Approximately 85 to 90 percent of the species are conserved in the animals, with some key differences. Filamentous bacteria control the entire IL-17 allergic and inflammatory response in mice. Humans do not have those organisms, so the humanized mice are a powerful tool with some limitations. So far, no researcher has taken a gnotobiotic mouse and transferred urine, although work is now under way to make such as transfer to determine the phenotype when urine alone is transferred.

Dr. Kimmel asked for an elaboration on the issue of immunologic response. He asked if the gut could be compared to the bladder, which is immunologically privileged, but there is not much of a lymphatic analogue. Dr. Seed stated that people with follicular cystitis appear to have spleens in their bladder, with a substantial immunological capacity. There also is a substantial capacity for converting to an adaptive immune response. In his work on the serologic response of women, his team produced complete unbiased display libraries with right different E. coli genomes representing 93 percent of the antigens present. Women who experienced their first cystitis episode had a dramatic immunologic response against the infection, clearly involving multiple levels of adaption. It may be that humans are constantly developing an adaptive response to the bladder's constituents.

Dr. Hewitt noted that a whole plexus of lymph nodes surround the bladder, and there are always simple lymph nodes at kidney. Dr. Kimmel noted that this does not necessarily mean immunoglobulins can pass through tight junctions.

Dr. Kim asked how the microbiomes could be identified. Dr. Seed responded that there are several ways. For example, some researchers have taken everything from organisms that are associated with particular syndromes. Researchers are reverse engineering the genome to identify what parts require nutrients. In other cases, researchers are developing specific ways of depleting a population of organisms, taking Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) designed against essential metabolic components to deplete specific types of organisms. Taken out of context of the community partners, a microbiome will behave differently. Reductionist studies are fine, but at some point it is necessary to expand back to a complete microbiome. Dr. Williams commented that the situation is akin to an ecological community that evolves to its environment. Very subtle changes in the environment foster community changes in response. Dr. Seed agreed that ecological principles must be employed and applied to new problems.

Dr. Barasch commented that one role of the urine microbiome might be to exclude more harmful bacteria. Dr. Seed concurred. In spina bifida studies, the hypothesis is that more protective microbiomes or pathogens have found their way into the microbiomes. That is a

starting point for considering the variables that must be examined more closely for their mechanistic contributions.

A participant commented that he was intrigued by the diversity between individuals and the organisms that they harbor, and yet the metabolic profile could be similar. He asked about the future path for better understanding metabolic profiles. Dr. Seed responded that the research clearly will involve a number of areas. The expressed genome of the microbiome will have to be studied. Currently, the resolution of proteomics is not nearly as great as for transcriptomics. On the host side, it will be necessary to understand proteomics and metabolomics, and very robust maps will be needed to cross-map the properties. The bigger challenge will be implementing those steps, with various hypotheses and players. It is a fast-moving area in which questions are being explored, such as how to create synthetic or derived communities and return them so that phenotype changes can be measured in hosts. In orthologous groups, there is incredible conservation across different microbiomes, microbes, or functional groups. The question is whether those are functionally expressed. Regarding microbial ecology, the dairy and agricultural industries have been conducting a lot of microbiome research because of an interest in how ruminants can make high-quality milk. Industry studies take advantage of the bioreactor idea to map the tools for moving forward and how to interpret the data.

Role of Proteomics
Dr. Jon Klein, M.D., Ph.D., University of Louisville, Louisville, KY

Dr. Jon Klein, fascinated by the term “urinology,” sought the earliest reference to the term and found it in the Boston Medical Surgical Journal. Further describing the historical aspect of the issue, he presented an illustration of Constantine the African, a Muslim who became ill after traveling to Italy. Constantine was seen by the best physicians in Salerno, a center of Italian medicine, and was shocked that the physicians did not ask to see his urine. He studied as a physician and then returned to Salerno to instruct the physicians in the proper approach to examine urine to diagnose disease. His knowledge was far more advanced than that of Europeans at the time. Five hundred years later, Ulrich Pinder created the urine wheels, with each color of urine corresponding to a disease.

In the early 1990s, researchers began developing different ways of looking at urine, specifically proteomic methodology and urine studies. Dr. Klein provided a short tutorial on proteomic methodologies, followed by cases in which proteomics was applied to urine studies, and then addressed the question of whether it is possible the kidney communicates with the bladder.

The first challenge with proteomic analysis is protein extraction, which involves the removal of proteins from their biological matrix. A variety of approaches are possible. Proteins can be physically disrupted with sonication, chaotropes, and detergents, or they can be milled. The proteins must be separated in a consistent and reproducible way. In the past, two-dimensional gel electrophoresis was used to separate proteins, but now liquid chromatography is commonly used to separate them using their chemical and physical properties. Previously, fluorescent gels were used to quantify proteins, but now mass spectrometry (MS) is used to quantify the proteins directly or label them with tagging agents that enable comparisons of samples across different groups. Mass spectrometers are used to identify and then quantify the peptides.

There are barriers to studying urine with proteomics and mass spectrometers. One challenge is that a broad, dynamic range of protein expression in urine above 14 or 15 logs is available, and the instruments do not exceed 14 or 15 log detection. Abundant plasma proteins hamper the study of elements that are of interest. One approach to the problem is to use affinity purification removal systems. IgY columns can be used against the 16 to 23 most abundant proteins from the urine, which allows the dynamic range to be compressed and provides a deeper view of the proteome.

Dr. Paul Tempst published studies in which he examined the degradation products of proteins to make inferences about the biology of the intact proteins as another way to circumvent the dynamic range challenge. When examining the low molecular weight fraction, what is being viewed are peptides less than 20, 10, or even 5 kilodaltons. Intact proteins of interest could be present, as well as so-called “conditionally unique” fragments, which means that the pathology itself causes the fragmentation of the proteins. Ladder-like peptides can be arranged, with each one slightly shorter than the next. These commonly are found in both plasma and urine. Dr. Klein’s laboratory has published papers on biomarker discovery of low molecular weight peptides. Disease- or organ-specific intact low molecular weight mediators can be found when examining the low molecular weight proteome, called a peptidome. Low molecular weight fragments that reflect the rate at which diabetic nephropathy progresses can also be identified; these fragments can provide lessons about protein expression in the kidney.

Dr. Klein’s laboratory has developed a scheme for processing urine for proteomic studies. The work product is available online at the website for an NIDDK workshop held 5 years ago. In urine sample handling and processing, the guiding idea is that information is contained in each fraction and collected. The researcher’s job is to preserve that information so that proteomic tools can be used to obtain the broadest possible impression of what the urine means in a pathological condition. The protocol first describes a soft centrifugation. From the clarified urine, exosomes are isolated and banked. The exosome-depleted urine receives ultra-filtration to achieve the separation state between the peptidome and the proteome. At that point, the remaining urine can be concentrated and desalted. Dr. Klein described

the work as a unified vision for urine to ensure that necessary information is collected and stored in the biobank.

Extensive work has been conducted on quantifying proteins and peptides, under three basic approaches. One uses a stable isotope labeling method using an isobaric tag for the peptides, allowing multiplexing and comparing abundances across different experimental conditions. The second approach is dynamic spectral counting. Peptides at higher abundances—the number of unique peptides are counted and adjusted for their molecular weight—tend to be proportional to the abundance. It is an informatics approach, as opposed to chemical labeling in the first approach. The third method is simply to examine the characteristics and features of the spectra themselves. Adjusting the base of peak and other approaches can be used to normalize the spectral characteristics. This unlabeled approach is used commonly. When the three are compared, the unlabeled approaches have become the mainstream in proteomic analysis.

Dr. Klein described a paper he authored that became the first proteomics paper published Kidney International in 2002. The publication identified only 33 unique gene products using acetone precipitation and 25 using ultracentrifugation. Many hydrophobic proteins were identified in the ultracentrifuge group, but the question was never asked why so many more membrane proteins were seen when the urine was ultracentrifuged. Last month, a paper was published in the Journal of Proteomics that identified 3,429 unique proteins, most of them contained in vesicles and most possessing hydrophobic residues, while the remaining were distributed through a different series of ways in which the urine was divided.

A publication by Dr. Mark Knepper of the National Heart, Lung, and Blood Institute in 2004 identified highly hydrophobic proteins in urinary exosomes, which are produced intracellularly. It is now known that a whole spectrum of microparticles is released by kidney cells. The work is remarkable, but the limitations of the mass spectrometer at that time did not allow Dr. Knepper to see a number of proteins of great interest in exosomes. Many are extracellular secretions, cytoplasmic structural proteins, and related to exosomal trafficking. The surface of exosomes in the urine reflects the portion of the plasma membrane of the renal cell from which they were derived. A 2014 Circulation Research paper showed that exosomes contain adhesion molecules that allow them to bind to other cells and then be endocytosed and transfer their contents, possibly altering that cell's behavior.

Dr. Klein noted that urinary microvesicle contents might alter other cells' behavior. The microvesicles contain a wide variety of growth factors, vascular mediators, and inflammatory modulators. Virtually every portion of the complement cascade is contained within exosomes. A 2013 paper from the Journal of the American Society of Nephrology (JASN) used a model of renal fibrosis to demonstrate that the number of exosomes increases dramatically when the kidney is damaged, and the exosomes contain large amounts of TGF- mRNA. Coculture experiments demonstrated that fibroblast cells placed in a hypoxia chamber also produce many exosomes. Furthermore, culturing 3T3 cells with exosomes shows that proliferation increases. A different JASN paper from 2014 looked at the exosome contents and then mapped the networks in the exosome. The researchers found a significant number of proteins that were either bactericidal or bacteriostatic. The publication suggests that exosomes placed directly into a bacterial culture cause lysis of bacteria because of the bactericidal contents. Dr. Klein's laboratory conducted a similar experiment culturing exosomes derived from patients with type 1 diabetes who have normal protein excretion with a proximal tubule cell line; the experiments demonstrated a biological effect simply through coculture of exosomes with the normal cells.

In summarizing his presentation, Dr. Klein stated that urine proteomic analysis is aided by separating urine components containing different information, such as the exosome, peptidome, and proteome. The comprehensive urinary proteome contains approximately 3,000 distinct gene products. More than 1,000 proteins that affect cell-cell interactions have been found in urinary exosomes. Changes in urinary exosomal proteins can alter cellular behavior, as shown by the studies discussed. Studies of exosomes in tumors show that this is a form of cell-cell communication, so it is not a significant leap to say that urinary exosomes may also engage in cell-cell communication.

Discussion

Dr. Kim asked if the size and contents of exosomes vary, and what method was used for isolation and collection in the study. Dr. Klein responded that the researchers used a two-cushion density gradient and selected for 600 to 200 nanometers, which is very broad. One criticism of some publications is that microparticle or microvesicle contents are so heterogeneous that many preps could also containapoptotic bodies.

Dr. Barasch asked if an examination of diverse kidney damage coupled with information on the exosome population could produce a straightforward diagnosis. For example, if a patient has proximal tubule or other damage, could that be mapped back to diabetes glomerular damage? Dr. Klein answered that no one has yet conducted a comparative analysis of different renal damage models to look at the exosome content. However, when his laboratory looked at diabetic nephropathy, researchers saw more changes in exosomes derived from proximal tubules than anywhere else.

Dr. Star asked about the methods to separate out different exosomes. He noted that good antibodies are not available, although there is one marker of glomerular damage. Dr. Dmochowski asked for any thoughts about bladder proteins. Dr. Weiqun Yu asked about secreted

protein. Dr. Klein suggested that it might have been beneath the level of detection.

Dr. Hewitt noted that 5 years have elapsed since a meeting was held on processing urine. Better processing equates a high-quality exosome. The problem is that there is no reasonable high-throughput method of isolating exosomes. Researchers need to develop a methodology to isolate exosomes before fractionation. Dr. Klein said that several kits are available on the market, but they do not work well. Dr. Klein has a patent on a filtration device.

Dr. Hoshizaki asserted that scientists know exosomes are produced by kidney cells in response to damage. They contain contents that can change cellular behavior, but it is unclear if they actually have a biological role. They might be useful as biomarkers to point to damage occurring, but if they have a biological role, why are they there? Dr. Klein responded that exosomes appear to have a role in the transfer of RNAs and microbiological fluids to allow cell-cell communication. In the prostate cancer literature, exosomes play a role in the extension of the tumor and activation of other cells. Dr. Hoshizaki wondered if the exosomes were trying to communicate with other cells downstream. Dr. Klein said that the law of biological parsimony suggests that some mechanisms are conserved. Dr. Hoshizaki added that this mechanism might provide a way for the kidney to communicate with the bladder. She suggested that the participants consider what experiments could affirm that a message was received by the bladder.

Dr. Kim described findings in oncology relevant to the issue of communication between cells. In concept, normal cells can receive signals from cancerous cells, activating transcription pathways. Normal cells receive oncoproteins or genetic material transferred in vesicles and then increase proliferation and invasion.

Dr. Chris Ketchum asked if Dr. Klein or others ever saw small functional fragments of organelles, such as mitochondria, in the exosomes. Mitochondria might be able to traffic from cell to cell and engage in repair work. Dr. Klein responded that he had not observed such mitochondrial fragments or objects. Specific mitochondrial proteins can be identified, but not necessarily their provenance.

Dr. Star stated that, whether functional or not, the Tamm-Horsfall glycoprotein forms networks in the urine. Dr. Klein noted that the Tamm-Horsfall glycoprotein can be seen either as a source of information or as a barrier. It is the abundant protein in urine and expands the dynamic range. The networks seem very dynamic in that they have biological channels and activity. In different disease states, the tendency of the protein is to form oligomers or to change its glycosylation, which seems to be associated with pathological conditions. Dr. Star asked if constituents could be transmitting information. If exosomes contain signals, the encapsulating meshwork becomes an interesting and complex system—an exosome buffer.

Role of Metabolomics
Jayoung Kim, Ph.D., Cedars-Sinai Medical Center, Los Angeles, CA

Dr. Kim, who uses metabolomics as a tool for studying urine in pathologic conditions, explained that her presentation would address urine, metabolomics in general, the urine metabolome, current efforts to develop a human urine metabolome database, and case study examples using urine metabolomics. Urine's composition is 95 percent water together with small amounts of ammonia, sulfate, and other constituents. Urine was formerly considered a waste and also sterile, neither of which is true. For the purposes of the meeting, Dr. Kim reiterated the question of whether urine plays an active role in regulating bladder biology. Urine is an ideal biomedium to monitor the bladder's condition. It is readily obtained and available. The ease of collection allows for serial sampling to monitor disease and therapeutic response. The body fluids that are most proximal to a disease site often can provide a source of informative biomarkers. Urine-based monitoring for bladder condition therefore is the most attractive strategy among other biofluids-based methods.

The metabolome is very much affected by the genotype and microbiome environment. Patients and their conditions can be classified using their phenotype and metabolome signature. Targeted metabolomics is a quantitative measurement analyzing a few metabolites and is used for the validation phase of a study. Metabolic profiling is used for selected metabolites. Metabolomics can encompass a broad range of metabolites and metabolic fingerprinting that enables the classification of samples.

Dr. Kim presented a general scheme for the urine-based metabolomics process. Ideally, urine samples are used as a diagnostic for disease or responses to therapy. General approaches to examining urine involve high-throughput extraction and analysis from urine samples. The aim of data processing is to associate the peaks and spectrum from metabolomics instruments with disease and responses of the patient, and, ultimately, to provide a disease diagnosis. Metabolomics information can be used in the patient's treatment, providing a personalized medicine approach.

Dr. Kim reviewed how metabolomics can be applied to urine biology research using analytical techniques and data processing. There are two analytical techniques, nuclear magnetic resonance (NMR)-based and mass spectrometry (MS)-based, each with its own advantages and limitations. The NMR approach has minimum sample requirements, possesses a quantitative ability, and provides structured information, but its sensitivity is less than that of the MS-based approach. For analysis, a number of techniques are available: liquid chromatography-mass spectrometry (LCMS); gas chromatography-mass spectrometry (GCMS); NMR; principal component analysis;

orthogonal partial least-squares discriminant analysis; and partial least-squares discriminant analysis. A combination of these instruments should provide a more targeted metabolomics signature. Work is ongoing in different cohorts, as well as institutional studies, using a targeted metabolomics approach. Data processing and metabolite identification require many databases. Dr. Kim briefly described the Human Metabolome Database (HMDB).

The workflow for metabolic profiling involves sample preparation and pre-processing. The urine sediments are removed through centrifugation and separation. The NMR, GCMS, or other MS instruments can be used. Coverage varies with different instruments, so it is helpful to combine NMR and MS analyses to provide a broad range of information that covers the metabolite profile. Pre-processing, peak detection, and deconvolution must be conducted. Pattern recognition is used to detect simple differences in patients. The metabolomics peaks are identified, and after independent cohort validation, the biomarker candidates can be confirmed.

Analytical challenges exist, however, in analyzing urine samples compared with other fluids, such as serum. Urine has wide variations in its ionic strength, pH, and osmolarity, particularly under conditions of physiological stress, diet, exercise, medications, health condition, and environmental exposure to pollutants. Care must be taken in interpreting metabolomics data.

Recently, Dr. Kim collaborated with Dr. Oliver Fiehn of the West Coast Metabolomics Center, University of California, Davis, on a review paper that focused on metabolomics chemistry, biology, and informatics. All three components are needed to reach a conclusion about the meaning of the metabolomics data. A variety of urologic samples are being analyzed, including tissue and urine. The collaborative work will create a data bank to benefit the research community. The BinBase library contains a great amount of information linked to protein expression information. It also can link directly to the pathways to enable consideration of the metabolite biology.

Dr. Kim provided several examples of analyses. The quadrupole–time-of-flight (QTOF) MS, which is used to identify unknowns, is useful for analyzing urine because it is a system that is specialized for hydrophilic samples. For data acquisition, several instruments are available. Because researchers use many different types of platforms and instruments, the question arises as to how all of the information can be normalized. An effort to harmonize data involving multiple laboratories working to integrate data will assess more than 1,200 target compounds in the database.

Adequate metabolomic databases have been established, but none are based in the United States. Researchers are working to accumulate metabolite information for the MassBank of North America (MoNA), which is inspired by and collaborating with Japan's MassBank. MoNA is supported by the National Science Foundation, as well as the NIH Metabolomics Consortium. The HMDB is the gold standard in the field of metabolomics. HMDB 3.0 now contains detailed information on more than 40,000 metabolites, representing an expansion of nearly 600 percent. Metabolites are divided into two types: "detected and quantified" and "detected (not quantified) and 'expected' metabolites," or those for which biochemical pathways are known or human intake or exposure is frequent but the compound has yet to be detected in the body. The pathway information showing the metabolites' link to proteins and disease information is available. Another approach is the Urine Metabolome Database (UMDB), which contains data on 50 NMR metabolites identified in urine.

Dr. Kim compared NMR with other spectrometry-based instruments. In one study, NMR provided information on 200 compounds, 100 of which were unique. GCMS produced 179 compounds, 89 of which were unique. These differences must be considered in designing a metabolomics research study.

Dr. Kim spoke about how researchers can use metabolomics analysis to understand the biology of the bladder. Urine has many metabolites. Some can be derived from the host, but others are from the bacterial microbiome. The different signatures cannot be distinguished clearly at this point. When in the bladder, metabolites crosstalk between the bladder and urine, with influence occurring in both directions. As a study example, Dr. Kim described research in which urine samples were obtained from controls and IC patients with severe symptoms. The NMR-based metabolomics study explored several questions, including whether urine sediments could be used for diagnostic purposes, the role of urine in bladder sensitization, how microbiomes and bacteria can communicate with the human bladder (host), and the phospholipidome's biological role in normal urine-derived extracellular vesicles.

Discussion

Dr. Barasch asked if MS preserves or destroys phosphorylation, sulfation, and other modifications in small metabolites. Dr. Kim responded that MS can be used to profile phosphorylation, acetylation, and other modifications of proteome. In response to a question from Dr. Lysanne Campeau, Dr. Kim responded that increased DNA hypermethylation at CpG island reduces gene expression. A great correlation was obtained when gene expression and methylation patterns were compared using different analysis methods including omics combined with computational approaches.

DISCUSSANT RESPONSES
Moderator: Tamara Bavendam, M.D., M.S., NIDDK, NIH, Bethesda, MD

Dr. Warren Hill

Dr. Hill offered a personal anecdote, noting that many individuals are likely to have experienced feeling the need urinate but then finding that relatively little is voided. He has noticed this in himself from time to time. At other times, the usual substantial amount of urine is voided. Sometimes the feeling of urgency is quite strong, yet there is not a resulting full void. Dr. Hill wondered if communication between the urine and afferent nerves is occurring, perhaps from something eaten – capsaicin in a nice Thai or Indian curry for example - or having more coffee than usual. He speculated that anyone interested in the bladder likely takes personal notes on his or her own behavior to figure out what is happening.

The most striking information he heard during the meeting's morning sessions pertained to the interaction between the microbiome and the function of an organ, such as the gut. The comment about the microbiome of the gut affecting neural circuity so as to alter the gut's motility is important because many bladder diseases are involved with altered motility, including underactive and overactive bladder and strong feelings of sensation. The new idea regarding communication, whether from microorganisms in the bladder or from constituents arriving from the kidney or from the urothelium's surface, is worth exploring.

An issue not yet discussed is the likely secretory components, including chemicals, metabolic components, and proteins being secreted from the urothelium itself. The urothelium is a highly sensitive structure and is known to secrete adenosine triphosphate (ATP), nitric oxide, and acetocholine, which are very powerful transmitters. Whether the other constituents identified in the urine through proteomics and metabolomics are in some cases deriving from the urothelium itself is a question. One challenge is the complexity of urine in discriminating between kidney and bladder contributions. Dr. Hill has had a strong interest in purinergic signaling in his own research. The bladder itself secretes significant amounts of ATP and presumably uridine diphosphate (UDP) and other constituents as well. Whether the kidney could be sending purinergic signals to the bladder in the vehicle of urine is a compelling idea.

Understanding of purinergic signaling has evolved through studying ectonucleotidases that break down ATP and UTP into bioactive metabolites. Purinergic signaling is likely to be more localized than diffuse because of the presence of those enzymes. The urothelium, at least within the lateral and basal membranes of intermediate and basal cells, has ectonucleotidases present. So far, however, none have been found on the surface of the apical membrane of the umbrella cells. Because no enzymes have been found there, one possibility is that ATP and UTP could be long-lived, long-acting molecules in urine.

The gut motility findings are an interesting analogy for bladder motility. Another interesting concept was the idea of the microbiome affecting barrier function. Dr. Mark Zeidel has studied in detail the barrier function of the bladder epithelium. Many hypotheses circulate about various bladder disease processes that may be initiated through a breakdown in barrier function. This raises the question of whether the tight junctions or uroplakin structures on the apical membranes of the umbrella cells are compromised. Also, a question arises about whether bacteria and the microbiome present in close proximity to the bladder surface might in certain circumstances compromise the barrier and initiate a series of perceived or actual symptoms. If potassium leaks across the barrier, afferent neurons within the urothelium are likely to be depolarized and send pain sensory signals to the brain.

Dr. Hill mentioned several experiments conducted by his laboratory germane to the Think Tank. With the idea that urine might be affecting bladder function, the kidney is thought to produce an osmotically enriched solution with widely varying pH. To examine the question of whether changes in bladder function are observed if urine composition is altered, one approach is to use cystometrograms (CMG) in mice or rats. Rather than infusing the bladder with saline, Dr. Hill's laboratory explored whether infusing the bladder with 2 molar (M) urea—the usual osmolality of mouse urine—would change the CMG. Disappointingly, no changes were observed. The experiments are worth pursuing with more rigor, but there is always concern about the potential for bladder damage by inserting a catheter and the insertion point being a source of possible leakage thereby stimulating a response. Finesse and technical expertise are necessary for this type of experiment to work.

Dr. Roger Dmochowski

Dr. Dmochowski commented that urine bioactivity cannot be considered without examining the storage mechanism. The renal microbe architecture is critical. Researchers need to better understand the urothelium and its importance from a standpoint of secretory products. Autocrine and paracrine factors should be considered. Assuming the stability of the urinary environment, modulating the urinary environment factor could change the entire milieu.

Dr. Dmochowski concurred with the critical importance of standardizing testing methods. He noted that some of the merits of nerve growth factor as a potential biologic method to assess disease were discredited because scientists were not using standardized methods.

Dr. Dmochowski emphasized the importance of the microbiome, especially in terms of urothelial function. Bladder diastolic function is dormant until a systolic contraction occurs. The bladder is passively accepting urine most of the time. Years ago, it was thought that micromotion heralded the start of an unstable bladder. The urothelium itself is important in the diastolic function as its stretches and

releases various neurotransmitters, stabilizing the filling process and improving vascular supply. Hypoxia and ischemia were not reviewed, but are an important aspect of the discussion. The point also was made that new receptor types are being identified and indicate parallels between the lower intestine and urinary tract. They were found to be more similar than dissimilar.

Dr. Tony Buffington

Dr. Buffington commented on the question of whether evidence exists that urinary constituents play an active role in bladder homeostasis, dysfunction, or disease. Domestic cats are a natural model of non-ulcer interstitial cystitis (IC). The MAPP network studies show that half of IC patients have comorbid disorders, and many (in my experience most) IC cats exhibit comorbidities. In an NIH-funded study, his laboratory obtained cats with bladder dysfunction from veterinarians and conducted in vivo and in vitro studies (reviewed in1). Initially, the laboratory regarded IC as a bladder disease, and with Dr. Zeidel's assistance, the researchers examined the urothelium. Dr. Buffington explained that the same week that his laboratory published a paper with images showing the cats' urothelium pathology,2 another paper was published with identical pathology images from stressed mice left in a vivarium with the lights on.3 The cats ultimately recovered: It was not just that their voiding dysfunction normalized, but their voiding function and everything else that was wrong with the cats also improved.4 Dr. Buffington and his colleagues were able to show in clinical trials and laboratory studies that a change in the animals' environment also could cause recrudescence of their signs, which appeared stress-responsive.4,5

Dr. Buffington described his experience with cats because one of Dr. Seed's images showed the microbiome affecting everything else. There are two camps of researchers, one thinking that disease is bottom-up and the opposite thinking it is top-down. In his view, IC is most commonly a top-down disease, but this cannot be discerned in the patient. Dr. Buffington emphasized that the organism is a community that responds to the environment. The primary therapy for LUTS in cats is environmental modification. In this situation, the CNS is the primary driver, not the bladder, even if some interaction is occurring. The same experiment cannot be conducted in humans.

Dr. Buffington acknowledged that the overall diet of an animal or human might make a difference in bladder function, although studies evaluating individual ingredients (e.g., acidic foods) have proven inconclusive. For example, when the urine pH in patients is modified in IC patients, no changes in symptoms are observed. Blinded protocols are needed to test the effect of diet on bladder function. Dr. Bavendam acknowledged the vast difference between patients' experience and explanations. Symptoms can be modified based on patient perception. Dr. Buffington added that as a nutritionist, he worries when patients may not not eat well because they have become afraid of food.

Dr. Lysanne Campeau

Dr. Campeau reiterated that urinology involves studying the constituents of urine and how they affect urine biology. From her perspective, the question is: Where are the constituents coming from? Urologists focus on the bladder, but constituents also are generated in the kidney. This interplay is expected with many systemic diseases, such as metabolic syndrome and other diseases that show an effect at the kidney and bladder level.

Dr. Campeau acknowledged the Think Tank presentations asserting the effect of diet, drugs, and diuretics on urine constituents. The exact nature of the composition remains unknown. What are the constituents that matter? Ions? pH? Lipids? Proteins? All of these different components likely have an effect. The question that interests her is: What do they actually do? Are they markers of diseases? Several different publications have explored the metabolome from a perspective of not only avoiding dysfunction, but also affecting the urothelium, nerves, and other functions. The issue is worth examining.

Dr. Campeau referred to a talk about the microbiome at a local conference, which made the interesting point that the microbiome is not the result of, but might be the cause of, many pathologies. Current research is exploring how the microbiome might cause metabolic syndrome. Dr. Campeau's particular interest is the Krebs cycle and how intermediates may differ in pathology depending on the conditions in the urine.

Dr. Lori Birder

Dr. Lori Birder explained that her research group studies the epithelium using a variety of models. One of the best chronic models is feline IC. Spinal cord injury and stress models also are known to affect urinary function. Urothelial cells express almost all hormone receptors, yet researchers still do not understand what is released when the cells are stimulated and where the signal travels. A major area of interest for Dr. Birder and colleagues is ATP. Cells release many substances. Her group is exploring what cells release, how they release it, when they release it, and how it affects bladder sensory functions. They have become interested in comorbid disorders. Comparisons can be made between the gastrointestinal, genitourinary, and pulmonary systems. For example, Dr. Anthony Ford's P2X3-antagonist drug works well for IC, overactive bladder, and chronic cough. Afferent epithelial and smooth muscle interactions are closely related.

Many epithelial cells express the same receptors and release the same factors. An important question is why some patients are differentially sensitive to environmental toxins and other exposures. In one study, researchers gave collected urine from individuals treated with antimuscarinics and instilled the urine in rats with hypersensitive bladders. This decreased the rats' bladder hyperactivity. Epithelial cell exposures can affect stress hormone levels.

Dr. Birder also is interested in aging; risk factors include ischemia and hypoxia. Models that disrupt the epithelial barrier's integrity alter the cells' response to perturbation. Senescent and signaling functions are different; bladder constituents, including cell-cell interactions, are affected. Responding to a question, Dr. Birder said that it is unknown if urothelial cells release exosomes. Urothelial cells synthesize and release ATP and growth factors.

Dr. Williams asked if the mechanism of exosome production was known to be random or programmed. Dr. Klein responded that endocytic vesicles are formed in cells, and portions of the vesicles enter into endosome trafficking. Some of the endosomes are then packaged into multivesicular bodies, comprising individual exosomes in sacs. Exosomes are different sizes because of different pathways. Microparticles result from a simple budding process, whereas exosomes are generated from within the cell. With so-called apoptotic bodies, cells seek to restrict proteins that might serve as pro-inflammatory agents or stimulate the immune response as the cell is dying. Dr. Hewitt added that apoptotic cells also send signals to cells nearby, either fortifying or warning them. Dr. Klein noted that they are referred to as exocytic particles; there are three distinct particles in lipid biolayers that have three distinct pathways. Dr. Seed added a fourth pathway, noting that outer membrane vesicles in budding yeast are well described. Data on enterotoxic bacteria show that the toxin is more potent as an outer membrane vesicle than as a free toxin. Exosomes can deliver nucleic acid material and metabolites and can target cellular receptors on a host cell.

Participants discussed the question of why humans have such dilute urine compared with other species and considered possible evolutionary reasons. The reason, however, is unknown. Dr. Hewitt posited that there might be an evolutionary cost and value gained, perhaps as a function of size. Dr. Williams agreed that evolutionary geneticists believe that major losses or gains are not made without some cost or value. Although it can be called a "cost," if a change remains within the genome there is a value. Mammals have different abilities to concentrate urine; this might be a function of size. The Loop of Henle is very long in the horse suggesting more capacity for concentrating urine, however, horses are one species with dilute urine. Dr. Hill noted that one theory suggests that humans adapted to run long distances to chase game such as gazelles. This ability might be linked to fluid regulation. Dr. Kimmel noted that if the question pertains to urinary dilution, then it is relevant to Paleolithic humans, not modern humans, who are subject to society's influences. Dilute urine might have resulted from adaptation to a protein diet. Without urea—which horses lack—it is difficult to create a concentration gradient.

Dr. Stephen Hewitt

Dr. Hewitt explained that he does not work primarily on urine, so he presented a broad perspective. He proclaimed that it is self-evident that urine is a bioactive fluid. That acknowledgement, however, raised the question of why the discussion about urinology was not initiated prior to 2015. Dr. Hewett posited one possible explanation: The biology of urine was not considered previously because urine had been analyzed historically from a chemistry perspective, with science locked into a paradigm that regarded urine as a sterile waste product—not as a bioactive fluid. As scientists accept the idea of urine as bioactive, challenges are revealed. The Think Tank discussions have highlighted many good questions, as well as the lack of acceptable techniques, designs, and samples to answer them. Agreement in the field that urine is a bioactive fluid will foster the development of new techniques, experimental designs, and sample collection. Unique challenges exist, such as the need to separate the kidney and bladder contributions to understand the function of urine. Dr. Hewitt asserted the need for a robust discussion of where to go next to solve these challenges. He said that if researchers question physiology and adaptation with respect to evolution—rather than considering issues from only a human pathocentric view—answers will be generated faster.

Dr. Williams commented that medical experts often discuss "adaptation," but use the term in various ways with different meanings. For an evolutionary biologist, an adaptation occurs through generation-to-generation change. Dr. Hewitt commented that APOL1 was an adaptation protective against an environmental stimulus, but it resulted in unintended effects of chronic kidney disease in modern humans. As another example, Pima Indians have modified their diets; their evolutionary adaptations against famine are no longer relevant.

GROUP DISCUSSION

Moderators: Deborah Hoshizaki, Ph.D., NIDDK, NIH, Bethesda, MD and Tamara Bavendam, M.D., M.S., NIDDK, NIH, Bethesda, MD

Dr. Bavendam presented framing questions for the meeting discussion:

- Is there evidence that urine constituents play an active role in bladder homeostasis, dysfunction, or disease?
- Does urine allow communication from the kidney to the bladder?

- Is it worth pursuing the idea of urine as a biologically active fluid?
- What data are desired (and types of experiments necessary) to establish whether urine is an active fluid?

Dr. Hoshizaki summarized the key points raised during the Think Tank, which began with the audacious question of whether urine is a biologically active tissue. Dr. Hewitt had responded affirmatively, arguing that there exists a whole series of biologically active molecules in the urine. Dr. Birder stated that the urothelium possesses a repertoire of receptors, and conceivably the urothelium would be receptive to substances passing through the bladder. Dr. Hoshizaki asserted that the meeting participants had explored the role of urine, if any, in maintaining bladder health and homeostasis. That concept is linked to a larger question about whole-organism physiology and how organs communicate with one another. Are there mechanisms that allow tissues of the kidney, bladder, intestine, and prostate to communicate with one another? At a minimum, the organs could communicate through innervation. One question is whether the bladder communicates with other organs, in particular, the kidney. If most bacterial infections traverse through the bladder, does the bladder alert the kidney that a problem is arising?

Dr. Hoshizaki continued with her meeting summary, noting that Dr. Barasch indicated that intercalating cells could be receptor-specific to the bladder and make NGAL and other proteins in response to the bladder's signals. She and Dr. Bavendam both possess a positive view of urinology as another approach to thinking about bladder health. One overarching issue, however, will require defining the bioactive molecule profile of urine. Researchers will need to address such issues as identifying the microbes present in the urine and understanding the effect of diet on microbes, which could change the profile of biologically active molecules. Perhaps a second, overlapping panel considering biologically active molecules is needed. Although solutions are not yet available, the Think Tank discussion has served to outline the complexity of the challenge ahead.

Dr. Hewitt commented that Dr. Hoshizaki had broadened the question beyond the kidney and bladder. The discussion must encompass fluid and diet intake, as well as physiology, and how these factors modify kidney, bladder, and urine function.

Dr. Kimmel commented that, with regard to Dr. Bavendam's first question, he was impressed with the amount of data presented by the Think Tank participants. The first questions to be asked involve the quality of the evidence and the degree to which urine compositional changes affect bladder homeostasis or dysfunction. Complex answers must be explored. Dr. Hewitt noted that scientists have not conducted the appropriate experiments, which is the challenge. Dr. Kimmel emphasized the importance of understanding how much of an active role urine plays, because if it plays a small role in exciting receptors—one that does not have an important effect, cause a systemic reaction, or feed back to the kidney during an overwhelming septic infection—that must be ascertained.

Dr. Hewitt referred to the oncology example of GFR3 in transitional cells that increases the risk of bladder cancer. Oncologists were excited about the discovery, as it introduced a new question that had not been considered. More than just obesity is involved; diet and behavior are other components of risk.

Dr. Star commented on the issue of the evolutional advantage of bladder receptors. Brain receptors can sense changes quickly, for example. What about the location of the bladder requires signal reception? He observed that concentrating urine could trigger receptors. Dr. Star noted the interest in bladder cancer because environmental toxins are processed through the microbiome and kidney, then end up in bladder. The question is whether there are benign examples of bioactivity.

Dr. Hewitt commented that the bladder must be able to regenerate, given the high level of exposure to toxins. Another notable fact is that the bladder is the only organ designed to shrink to an empty orifice, then expand to hold 500 or 1,000 mL of liquid. The mechanical demands on the bladder are unique and require distinct receptors.

Dr. Dmochowski remarked on the limited autonomic understanding of organ function. Muscles and the bladder are not the same, partly related to the need for afferent connections with autonomic subconscious, as well as visceral, perception. The bladder used to be considered a strictly passive organ. The purpose of receptors in the physiology of the bladder is expected to reflect the need to optimize the storage function while also evacuating.

Dr. Buffington asked what happens when urine is diverted into the bowel. Dr. Dmochowski stated that there are differences in absorption in bicarbonate and fluid resorption between the colon and intestine. The classic teaching is that the histology of the bowel must be examined above the urinary diversion during surgery. If the colon is positioned in the urinary tract, changes in the transitional epithelium could influence the bowel. Dr. Kimmel commented that ileal or ureteral implants could produce the best evidence that urine is a biologically active fluid, and Dr. Dmochowski agreed that it would provide direct evidence.

Dr. Kim noted that an issue is whether urine is an active or a passive biological fluid. Dr. Hoshizaki stated that all meeting participants appeared in agreement that biologically active molecules exist in urine. The question is whether the biological profile plays any role in bladder health. Conversely, if there is communication from the kidney to the bladder, with the bladder then providing feedback to the kidney, there could be a signal from the bladder to stop making NGAL. The issue of whether urine has a biological role acting as a tissue

is not necessarily the view of Drs. Hoshizaki and Bavendam, but they wanted to generate as broad a conversation as possible. Dr. Hewitt noted that discussion had not addressed the ureter or renal pelvis. Many transitional cells are capable of influencing and participating in all of the processes, especially transitional cell carcinoma.

Dr. Barasch stated that the connection of kidney to urine to bladder is straightforward with regard to antimicrobials; clearly, there is an antimicrobial effect. He described several known processes that result in health outcomes and commented that identifying other pathways would be cutting-edge science. If the bladder were found to secrete various defensive molecules, that would change conversation quite a bit because it would signal an autonomous organ rather than an organ dependent on the kidney. Straightforward experiments could be conducted using turtle or toad bladders to see if proteins are secreted and determine if the bladder is an autonomous organ. If no factors are secreted, that suggests a kidney-to-bladder system. Dr. Yu and other participants discussed the possibility of urine's providing a signal and an approach to determining if the effect is active or passive.

Dr. Bavendam stated that there appeared to be agreement that biologically active molecules found in urine might positively influence bladder function. Dr. Hoshizaki added that exosomes contain materials with the potential to change cellular behavior, but the question to be answered is whether they change bladder behavior. Dr. Hewitt discussed data pertaining to FGF3 and how experiments to test the question could be conducted in a relevant system, and members remarked on a co-culture experiment that already is being performed.

Dr. Hoshizaki summarized the discussion by stating that the answer to the first question is that urine constituents do play an active role in bladder homeostasis, dysfunction, or disease, but the details are unclear. Dr. Hewett reiterated that the toolset to explore the question is unavailable. Dr. Hoshizaki noted that perhaps the answer to her question is unknown.

Dr. Bavendam referred to the second question: Does urine allow communication from the kidney to the bladder? Dr. Hewitt urged restating the question: Is urine a medium of communication? Otherwise, the question implies that the kidney is in charge. The bladder could be the initiator of communication.

Dr. Star raised the issue of nephrotic syndrome, and participants discussed what is known about the subject. Dr. Kimmel noted that studies of children with nephrotic syndrome indicate that the bladder is not a major locus of pathology. Physiologically, children do not complain of bladder pain or emptying problems, but complain because they are "puffy."

Dr. Campeau stated that the second question refers to the fact that whatever affects the kidney also affects the bladder in such systemic disorders as diabetes and hypertension. Urine might not be a medium of communication, but whatever affects the nephron affects the urothelium in a similar fashion. After participants briefly discussed data on several receptors, Dr. Bavendam asked again if it was worth pursuing the idea of urine as a biologically active fluid. She asked the participants whether they would advise developing the idea and whether—with all the information available and all the acknowledged limitations and barriers—continued activity is likely to yield knowledge.

Dr. Williams asked if it was a different question to focus on "urine as a biologically active fluid" rather than the bladder as a biologically active organ. Dr. Hoshizaki responded that urine was a conduit into the conversation. Ultimately, the real goal is bladder health. Dr. Hewitt suggested the important concept is whether the bladder can communicate to the kidney through the urine. With regard to the urine biome, ideas are changing rapidly, much as the understanding of the intestines has changed.

Dr. Seed commented that new associations and connections are constantly being discovered, disproving traditional dogma. For example, researchers thought the skin microbiome was benign, but there is a clear mechanism now demonstrating dermatologic pathology related to the microbiome. Dr. Williams stated that in his view, urine is not a biologically active fluid. It might contain molecules that can communicate with the bladder. The participants then discussed the meaning of "biologically active." Dr. Seed noted that microbes form social communities with specific structures, such as biofilms, which change in disease states. In a sense, the microbial community could be considered a tissue. Dr. Hewitt approximated that 75 percent of the higher-level microbial function observed in the intestine will likely be reflected at some level in the bladder. Symbiotes exist in the intestine and also might be found in the urine. Dr. Seed stated that the oral, vaginal, and intestinal mucosa are seen as sampling environments, in contrast with the bladder, which has been regarded as a noncommunicative organ. Bacteria, however, are able to penetrate the bladder and communicate.

Dr. Kimmel commented that an attractive aspect of considering urine as a biological fluid is that the kidney is incredibly responsive and functions as a homeostatic monitor. If the amount of salt or protein ingested is changed, urine reflects that change. Zinc and micronutrients are excreted in the urine, and all reflect the daily load. Asking if urine can influence bladder function brings together nephrologists and urologists because they want to know how the kidney functions and the bladder responds. It also introduces diet and environment interactions for a more holistic perspective. From this diverse information, a "big data" systemic model could be created so that the bladder is not studied in isolation.

Dr. Hoshizaki stated that given the complexity of organ crosstalk and changes in the profile of biologically active molecules—depending

on the time of year and other factors—one important question is the appropriate focus. For example, NIDDK considers whether to study how the normal system functions, or whether to regard the normal system as a control and focus on urinary dysfunction. The issue must be discussed explicitly to determine the direction NIDDK should take in moving forward, since there appeared to be agreement that NIDDK should advance the study of urinology. Dr. Hewitt, indeed, had suggested that a whole new toolset should be developed.

Dr. Hewitt commented that medical schools do not adequately promote physiology. Dr. Hoshizaki noted that the physiological approach focuses on how all of the pieces fit together, rather than on a single cellular pathway.

Dr. Williams commented that three elements of the discussion intrigued him: bacterial ability to communicate through the bladder, exosomes' apparent ability to communicate through the bladder, and molecules in the urine with corresponding tissue receptors. Partitioning those elements seems to be fundamental to pursuing a mechanistic approach. Dr. Kim agreed. Dr. Hoshizaki added that overlaying the three elements is the issue of how the bladder returns communication to the kidney and how the circuitry is maintained. Certainly the prostate and bladder always communicate, posing an additional layer of circuitry.

Dr. Marva Moxey-Mims suggested that the group focus beyond inter-organ communication through innervation. When kidneys are transplanted, for example, innervation is severed but communication remains, indicating that communication goes beyond innervation. Dr. Hoshizaki responded innervation is "under the lamppost," but she would be happy to consider issues outside of that focus. Dr. Hewitt stated that from a surgical perspective, physicians do not consider the bladder in terms of innervation, but rather from a physiological perspective. Surgeons tend to downplay that the bladder is a true nexus of innervation.

Dr. Kim noted that tissue samples and a biorepository would be helpful for research. Dr. Hoshizaki responded that necessary resources will depend on the research to be conducted. Good resources are available, including developmental bladder data and urothelial renewal in the adult mouse. Part of the process will be to frame the question and then determine if resources are available.

Dr. Bavendam stated that participants appeared to agree that some aspect of urinology is worth pursuing, but specific aspects should be defined further. She asked if that lack of tools was a limiting factor at this point and if further research should await tool development. Dr. Hewitt recalled a discussion from an NIDDK-sponsored meeting in August 2002 that convened a bladder research progress review group. At the time, NIH was conducting high-level evaluations of disease processes, and the bladder was one area of focus. The meeting produced a strategic recommendation that NIDDK should develop new technology-driven basic and clinical research techniques, including animal models (genetic knockout models), stem cell therapy, gene therapy, tissue engineering, functional imaging, biomechanical and micro-engineering technology, genomics and proteomics, and cell regulation. The discussion of bladder health in the context of physiology did not occur in 2002. The results from the 2002 meeting would be useful to the current discussion.

Dr. Barasch favored focusing on the idea of kidney disease and the uroepithelium and posited questions that could be the focus of research: Does the uroepithelium change in specific kidney diseases? Does bladder function change? It is known that a number of gene knockouts change urine composition and function. Which urine constituents (e.g., receptors, small molecules, proteins) activate bladder contraction and function is a second research idea. Whether the bladder communicates back to the kidney is a third idea. The role in infectious disease is clear, but are there other neurologic or cytokine signals?

Dr. Kimmel stated that all the questions were helpful in developing a research agenda. Funding opportunities could target teams including urology, neurochemistry, pathology, and animal model expertise to investigate bladder pathology in the animal models.

Dr. Hewitt suggested that should the meeting participants firmly agree that urine is a bioactive substance, a forum could be organized at the American Society of Nephrology (ASN) meeting to advance the discussion. Dr. Kimmel noted that ASN will not make its final conference decisions until April 2015, so there might be time to propose such a forum.

Dr. Williams commented that Society for Integrative and Comparative Biology (SICB) organizers could be approached for partnership opportunities. The Think Tank participants and evolutionary biologists could be convened to perhaps initiate a program for researchers to explore the evolutionary significance of some of the issues. Dr. Hoshizaki agreed that the SICB could be a good organization to contact.

Dr. Dmochowski cautioned that animal models often do not successfully predict human responses, particularly in IC. Participants discussed the potential usefulness of the diabetic animal model that combines nephropathy and neuropathy from different causes and potentially could provide additional avenues for research. The call for research could solicit an integrative approach to diabetic renal bladder disease that also accounts for the microbiome and the neurologic system.

Dr. Hoshizaki asked again if, given the complexity of the issues, research should start with basic investigation of how the various systems function, or if normal conditions should be used as controls to delve into disease models. Both approaches have advantages. From her perspective, studying disease when so much is still unknown would be challenging. Dr. Kimmel responded that those were administrative decisions about whether to take a top-down or bottom-up approach. Providing direction either way would be useful.

Dr. Hewitt noted that a classic reverse genetics approach on a verified model might work. It is, however, unclear if an appropriate model exists; the research community could be queried. He commented on the opportunity of studying autoimmunity: Effector cells from elsewhere in the body could be traversing through the kidney to the urine.

Dr. Hoshizaki added that a comparative evolutionary approach would provide a framework to move forward if mechanistic questions of how organs communicate with each other are of interest. If the question is focused on a disease situation, then a comparative approach is inappropriate. Actual questions will dictate the approach. Dr. Williams noted the difficulty in securing funding for comparative research. Dr. Buffington suggested starting with basic physiology before advancing to comparative approaches. Currently, scientists do not understand the extent to which molecules, exomes, or microbes are involved in crosstalk, and research tools are needed to characterize those factors.

Dr. Hoshizaki noted the participants seemed to support convening another meeting. Involving ASN would be a way to generate broader interest in the research community. The participants, however, have not fully articulated goals. Dr. Hewett suggested developing a white paper for the ASN meeting. Dr. Star added that a short summary of this meeting would be one outcome of the Think Tank. A second product would comprise data and examples of urine bioactivity. Dr. Star outlined a number of ways available to move forward. The approach could be to conduct basic research followed by disease-specific research. As next steps, he suggested forming a small work group to produce a white paper, which could be circulated to the broader participants for comment. Dr. Barasch volunteered to take the lead writing the white paper. Dr. Bavendam added that after the meeting notes are available, discussions will consider the next steps.

Dr. Star thanked all of the participants for their helpful insights in approaching a fascinating subject. The Urinology Think Tank involved innovative thinking about an issue that has been percolating at NIDDK for some time. Dr. Star concluded that participants agreed that the subject is worthwhile to pursue and foundational steps should to be defined to move the field forward.

Meeting attendees are not available at this time.