Epidemiology, Molecular, and Genetic Methodologies to Evaluate Causes of CKDu Around the World: Report of The Working Group from ISN International Consortium of Collaborators on CKDu.

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*Complete list of i3C meeting attendees provided in **Table S1**

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Twenty years ago nephrologists working in El Salvador and Sri Lanka described a progressive kidney disease leading to devastatingly high rates of death from kidney failure in young and middle-aged individuals [1, 2]. Other descriptions including documentaries and press reports brought CKDu to the world's attention, presenting the toll on working families unable to afford kidney replacement therapy [3]. Additional regions with a similar profile of kidney disease have since been discovered but there are few rigorous studies investigating candidate hypotheses. Research has been challenging due to political circumstances, the marginalized nature of populations afflicted, and the scarcity of personnel and funding.

With support from the International Society of Nephrology, a multi-disciplinary group of nephrologists, epidemiologists, and occupational health and environmental scientists have formed a consortium (i3C) to identify a coherent approach to studying chronic kidney disease of unknown etiology (CKDu). We previously presented recommendations for surveys to detect CKDu[4]; here, we provide recommendations for studies focused on investigating cause(s) (**Box 1**). Specifically, our goal was to consider the most promising approaches with respect to 1) designing field studies, 2) integrating molecular analysis of biosamples into CKDu research 3) protecting the interests of study participants, and 4) promoting collaboration.

Potential Study Designs

Table 1 outlines potential study designs for further field work to investigate possible causes of CKDu. Longitudinal studies of CKDu are thus far underrepresented and are a priority. Longitudinal studies will: 1) avoid the undefined lag between exposure and detectable kidney disease that leads to recall bias with self-report of prior exposures, 2) reduce misclassification of acute (unrelated) kidney injury as 'CKDu', 3) identify participants with declining kidney function but not yet with eGFR < 60 ml/min/1.73m^2, and 4) minimize survival (Neyman) bias caused by failure to observe individuals with very rapid declines in kidney function.

Another critical issue is choice of population (e.g., occupation-, clinic- or community-based). In addition to comparisons of exposure between affected and unaffected individuals within communities, study designs may need to compare exposure differences between communities with and without high prevalence of disease since many individuals exposed to the critical risk factors may not develop disease.

Investigators have pursued many potential etiologies of CKDu including heat stress, nephrotoxic metals, agrochemicals, infection, and genetic predisposition. Heat stress and nephrotoxic metals have been investigated in several studies of modest sample sizes, the former in Mesoamerican countries and the latter in Sri Lanka. Ongoing studies are using sophisticated techniques such as telemetry-based internal temperature measurements, accelerometer-based assessment of work rate, and mass spectrometry for heavy metal exposure. Newer studies are also evaluating agrochemical exposures by direct measurement, but data published thus far rely chiefly on self-reported, rather than measured exposure matrices. Other hypotheses, e.g., food-based toxins, have yet to be explored. Many studies were designed at the outset to study a single hypothesis, and thus do not take full advantage of extensive fieldwork performed. A well-designed prospective field study would integrate capacity for testing multiple potential exposures, including for direct sampling of select environmental exposures.

Molecular techniques and biosample choices

In parallel with the increasingly sophisticated techniques being applied to evaluate environmental exposures in CKDu endemic regions, techniques to assess biological materials at the molecular level may contribute to identification of potentially causal factors by 1) allowing precise testing for specific compounds suggested by epidemiology studies, and 2) enabling massively parallel, unbiased approaches that could identify unexpected potential factors (**Table 2**). Compelling associations between exposures and disease identified in epidemiologic studies must ultimately lead to further translational work to understand disease pathogenesis. This process is exemplified by Aristolochic acid nephropathy, where epidemiology identified a candidate risk factor and molecular studies established a causal relationship.

The optimal set of biological samples for analysis is not a straightforward choice. Since the kidney is central to clearing toxins from the body, blood levels of many compounds rise when kidney function falls, making causal associations difficult. Urine represents an easily obtainable, data-rich window into kidney function but its composition varies widely based on recent dietary intake, complicating standardization. Kidney tissue has been little studied to date, because biopsies are not routinely performed in several of the affected regions, because many patients may only seek care once symptomatic with advanced disease, and because when a clinical diagnosis can be made, tissue diagnoses may not change management. Despite the challenges, promoting local capacity (infrastructure, equipment, and skills) for kidney biopsies represents an essential step forward in CKDu research for describing natural history, and for studies such as epigenetics and pathogen detection where biological information may be kidney-specific. Acquisition of biosamples in CKDu requires inevitable compromises and may require use of integrated information from several biosample sources (Table 2).

Given the effort and expense of performing field studies to investigate CKDu, maximizing use of samples and data is of high importance. Groups performing field studies may exponentially increase the value of their own work by facilitating the incorporation of complementary technical expertise. All research teams will benefit by asking how the samples and data they collect today can be leveraged to provide value beyond their specific research questions now.

Ethical Considerations

International collaborators are conducting research in areas where governance structure for the ethical review is poorly developed, and where government instability and political pressure can create risks for both participants and researchers. Here we discuss three considerations highly relevant to CKDu research.

Community Engagement

Participants in international studies may have little formal schooling and cultural norms may include deferring decisions to community leaders. We suggest researchers go beyond the standard individual informed consent, and first establish mechanisms for information sharing and building trust, such as qualitative interviews, town hall meetings and study advisory boards composed of at-risk community members, patients, and caretakers. In one ongoing study, the researchers conducted *a priori* town hall meetings in partnership with local government officials[5]; these informed prioritization of hypotheses around water-based exposures, a primary concern for community members. In another, community members' feedback informed the consent process, especially on storage of biospecimens, since misunderstandings regarding use of biospecimens were common[6]. Thus even in resource-limited settings, core principles of community-based participatory research can be implemented to prioritize hypothesis testing, design consent(s) and define risk tolerance based on an understanding of community preferences and needs.

CKDu researchers are increasingly considering hypotheses related to childhood exposures. Research in children, especially if parents have low health literacy, requires understanding local laws and customs, and benefits from standardized procedures when obtaining pediatric assent[7].

Bio-repository

Investigators need to think through the goals and operational mechanisms supporting a biorepository long before starting to collect biosamples. Careful consideration should be given to whether an in-country biorepository is feasible (**Box 2**). Researchers should consider requesting "broad" consent for future use of their de-identified biomaterials beyond the specific initial reason for collection. Researchers should explicitly acknowledge and allay participant concerns about possible commercialization of biosamples[8]. Biobanking also poses risks for breach of confidentiality that could lead to social stigmatization and discrimination [9]. This is most common with genetic research, where genetic information is linked with the clinical data of the participant; the American Society of Human Genetics has made recommendations to minimize this risk.

Return of results

Returning results to research participants is a costly and time consuming task, especially in research settings where participants may not have access to mail delivery, phones, or email. Researchers may not appreciate participants' interest in the science in general and their individual results in particular. In our experience with several active CKDu field studies, receiving results can be a key determinant of whether a potential participant elects to enroll in a research study. We strongly endorse protocols where study personnel deliver results to participants and making clear when research results will not be returned, and why.

Enabling Collaboration

The importance of collaboration cannot be overstated in attacking a problem that spans multiple continents, includes many low resource countries, and focuses on a difficult to characterize phenotype. While the scientific challenges are enormous, the opportunities for making a major research impact are also great. Many of the obstacles to progress in CKDu are unique, while others are inherent to research in general where competition for funding is intense and career advancement is often driven by personal rather than team-oriented achievement.

Need for Observatory of Studies

The need for replication cohorts, for large sample sizes, for disseminating new technologies, and for squeezing the most out of every research dollar and sample suggests that CKDu researchers would benefit from a study database for CKDu. We would advocate for the simplest set of starting conditions. Toward this end the ISN has created a survey for CKDu investigators to register study question(s), location, and biosample collection plans. Eventually, this observatory might evolve into a central source of de-identified datasets, or repository for biological samples and/or standardized data collection instruments, though we believe that the requirements for this expansion in scope are significant and should not delay us from establishing an information resource.

Resources and Standardization

The literature on CKDu is wide-ranging with respect to geography and scientific focus. We view this diversity of focus and approach as an asset. We do not support a centrally-driven approach to CKDu research. But we believe that providing the means to share information about individual approaches is key to evolution of better individual approaches. Examples include molecular protocols, best practices for sample collection and storage, and sources for sample analysis of various types. This may grow to incorporate biological and chemical standardized reagents or even laboratories willing to serve as core facilities for certain assays. The nephrology community experience with creatinine determination, where measurements have become standardized by mass spectroscopy based calibration, suggests that a shared standards may help reduce noise in the data and align studies to facilitate data pooling. Standardization is especially important for techniques that generate non-hypothesis based data.

Sharing frameworks

Incentivizing cooperation may be the single most important factor in fostering collaborative progress. Among the most effective ways is finding ways to ensure that contributions to projects of all types are fairly rewarded. Multiple first and senior authors are one way to share credit, especially when one group is primarily responsible for a field study component and another contributes the essential analytic techniques.

Other avenues include consortia where contributions are defined prior to study inception. The ISN and regional consortia such as Consortium for the Epidemic of Nephropathy in Central America and Mexico (CENCAM) could have highly productive roles in energizing these collaborations. Following standardization of data collection instruments and laboratory assays, and implementation of sharing frameworks, a critical next step will be facilitation of the data pooling process—i.e., methods for storing, transferring, and analyzing data—that will help generate sufficient statistical power to answer questions that no single group can address.

Summary

The momentum building in CKDu research is encouraging. We have seen progress as measured by increases in the number of research groups involved, the size and diversity of studies, and the quality of publications. We still remain far from answers regarding the cause of the disease or ways to help people with CKDu. We have outlined

the major challenges for the field as a whole, and some specific recommendations from our group on how to move forward (**Box 1**).

Supplementary Material

Table S1: i3C Meeting Locations and Attendees.

Supplementary information is available at *Kidney International's* website

Disclosures

All authors declare no conflicts of interest

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Box 1. i3C Recommendations To Strengthen Investigations of CKDu/MEN Cause(s)

i3C Recommendations To Strengthen Investigations of CKDu/MEN Cause(s)
1) Pursue longitudinal studies, and make provisions for biorepositories.
2) Bolster capacity for kidney biopsies, for diagnosis and investigating pathogenesis.
3) Create an open access database of ongoing studies to enhance collaboration and transparency.
4) Partner with participants and community leaders in study design, and ensure return of study results to participants.
5) Build long-term relationships between international and local researchers that emphasize fairness, trust, and shared commitment.

Box 2. i3C Guidelines to Develop Biorepositories from Field Studies of CKDu/MEN

i3C Guidelines for Biorepository to Develop Biorepository from Field Studies of CKDu/MEN
1) Well-defined and consistent donor inclusion criteria
2) Appropriate informed consent for both planned and potential future uses (broad consent)
3) Standardization of collection procedures
4) Measures to ensure de-identification of samples and confidentiality of data
5) Effective storage of biomaterials to ensure viability
6) Review procedures for evaluating and processing researcher requests, avoiding duplication of efforts, and dispute resolution

Table 1. Potential study designs and applications to CKDu/MEN

Study Type	Potential population (s)	Potential assessments	Advantages	Disadvantages
Cross sectional				
Population-based	Representative sample	Prevalence, trends over time if repeated	Cheap, efficient	Limited data on cause; outcome misclassification (e.g., AKI as CKD, early disease as 'healthy')
Cross-shift	Farm workers	Task-based prevalence of AKI	Cheap, efficient	Acute creatinine changes may be unrelated to CKDu; need a large study to assess risk factors if incident AKI low; unclear generalizability since cases may represent subset of disease
Family pedigree	Families with multiple members with CKDu	Identification of genetic variants that cause susceptibility to CKDu	Focus on highest risk individuals	Labor intensive; low power to detect genetic variants of small effect size; outcome misclassification (early disease as healthy)
Case Control				
Known disease	Cases drawn from clinic, using case definition appropriate for tubulointerstitial disease	Exposures correlating with case status	Efficient; allows for comparisons to healthy populations	Reverse epidemiology [&]; any associations likely to be self-reported; large sample size to test multiple candidate hypotheses; possible need for multiple healthy controls
	Controls ideally age-, occupation-matched & possibly from endemic and non-endemic regions*			
Acute presentation	Cases drawn from hospital or clinic with symptomatic AKI	Exposures correlating with case status	Measured associations more likely	Recruiting controls; misclassification of unrelated AKI; cases may represent a subset disease
	Controls from hospital with other causes of AKI and/or occupation-matched without recent AKI			
Longitudinal cohort				
Chronic cases	Established cases of CKDu	Risk factors for progression of CKDu	Motivated population; understanding natural history	Defining established cases; limited extrapolation to instigating trigger
General population	Healthy participants free of kidney disease	Risk factors for incident CKDu	Measured associations more likely; more reliably able to conclude causality	Large sample size to test multiple risk factors; need to define time points for assessments; need to define incident CKDu; highest yield study only within endemic* populations; drop out *Case control designs nested within larger prospective study could limit analytical costs and maximize statistical efficiency*
Occupational population (e.g., Cross harvest)	Farm workers	Risk factors for incident CKDu	Measured associations more likely; high risk population	Subacute creatinine change may or may not link to subsequent CKDu; large sample size to test multiple risk factors; drop out limits rigorous ascertainment of CKD status

*If studies are performed only within endemic regions (i.e., regions with a high prevalence of disease), exposures may be near-universal, making it difficult to test for associations with disease [&] In cross-sectional case control studies, a surviving patient is much more likely to be recruited; thus an association may be protective rather than a causative factor

Table 2. Techniques for biospecimen analyses relevant to CKDu

	Purpose	Advantages	Challenges	Costs	Samples
Genetics	Define differences in individual or group susceptibility to disease. Where possible, this can facilitate identification of molecular pathways responsible for disease pathogenesis.	Genetic differences are fixed at birth so direction of cause-and-effect between genotype and measured variables is clear.	Multi-ancestry admixture and inter-relatedness in most CKDu clusters are more difficult to control for than in homogeneous, larger, more geographically dispersed populations.	Decreasing rapidly; Approximate costs of genotyping arrays $125-250, Exomes $300-600, Whole Genomes $1,000-2,000. Data analysis costs can add considerably.	Essentially any tissue. Whole blood and saliva are easy to obtain and yield large amounts of good quality DNA.
Epigenetics	Understand how environmental factors can modify expression of genes, often by acquired molecular alterations to the genome.	Genes and environment are both likely important factors in CKDu. Epigenetics is one major way these factors interact.	High-throughput genome wide tools lag behind other molecular approaches, though there has been much recent progress.	Methylation arrays: approximate cost $250-$650.	Unlike genetics, tissue selection critical. Kidney tissue would be optimal but difficult to obtain sample numbers needed for genome-wide statistical thresholds. Other tissues (e.g. WBC) may be useful for "fingerprinting" recent exposures.
Pathogen Sequencing	Identifying pathogens not yet known to cause kidney disease.	Can potentially identify thousands of known and novel pathogens.	Better for acute than chronic disease when viral sequences likely to be most abundant. Pathogen nucleic acid may not be detectable in easily accessible tissues.	Decreasing rapidly. Requirement for analytic resources may be high because methods are not straightforward.	Kidney tissue is optimal but urine or even serum or environmental samples may be useful in some cases. Timing of sample collection (acute vs. chronic) also a key factor.
Proteomics	Determine alterations in protein composition of various samples between health and disease states.	Tools have become remarkably sensitive in recent years. Potentially useful for biomarker development.	Sample quality is important and can be particularly challenging in low resource settings.	Widely variable	Urine**, serum.
Metabolomics	Measure differences in small molecules from various sample types in health and disease.	Can measure downstream consequences of many different types of perturbation (genetic, diet, toxic exposure, etc) or identify exogenous compounds.	Low GFR will cause widespread changes that are independent of cause of disease, especially in serum. Adjustment for urine concentration can be challenging. Uncommon exogenous compounds may be difficult to identify.	Widely variable	Urine**, serum. Mass spectrometry may be especially effective for identifying low abundance compounds (nanomolar range). NMR-based techniques are less sensitive (micromolar range) but in some cases offer more versatility across classes of molecules and offer quantitative readouts.
Analysis of toxins and metals	Detect presence of nephrotoxic elements and compounds.	High sensitivity detection of a wide range of toxins, including metals,	Ability to detect toxins depends on recent exposure in most cases.	Widely variable but testing across a wide	Urine**, serum, hair, nails. More stable adducts may be observed in kidney tissue, for

		agrichemicals, environmental toxins, and also metabolites after conversion of parent compounds by metabolizing enzymes.		range of exposures is invariably expensive.	example aristolochia-DNA adducts in aristolochic acid nephropathy. See above for mass spectrometry vs. NMR based techniques. In special cases, antibody-based assays (e.g. ELISAs) are available.

*Exposure monitoring using environmental samples (such as water, air, and soil) are not included in this table but remain potentially powerful tools for the study of CKDu. ** The choice of biological specimens for study and how to collect them are rarely as straightforward as they might seem. To illustrate some of the challenges awaiting the CKDu investigator, consider the example of analysis of urine. When do you collect samples: standardized for time of day and strenuous working conditions or "random" sampling for simplicity? How do you collect the urine: with protease inhibitor for proteomics, with bacteriostatics for metabolomics, with additives that preserve nucleic acids for pathogen detection? As techniques become more sophisticated the requirements for sample optimization increase. The best approach in any given situation depends on the specific questions that one wants to ask and the tools that will be used.