Published in final edited form as:
J Cardiovasc Transl Res. 2012 August ; 5(4): 491–508. doi:10.1007/s12265-012-9382-7.

Perspectives on Systems Biology Applications in Diabetic Kidney Disease

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Abstract

Diabetic kidney disease (DKD) is a microvascular complication of type 1 and 2 diabetes with a devastating impact on individuals with the disease, their families and society as a whole. DKD is the single most frequent cause of incident chronic kidney disease (CKD) cases and accounts for over 40% of the population with end stage renal disease (ESRD). Contributing factors for the high prevalence are the increase in obesity and subsequent diabetes combined with an improved long–term survival with diabetes. Environment and genetic variations contribute to DKD susceptibility and progressive loss of kidney function. How the molecular mechanisms of genetic and environmental exposures interact during DKD initiation and progression are the focus of ongoing research efforts. The development of standardized, unbiased high throughput profiling technologies of human DKD samples opens new avenues in capturing the multiple layers of DKD pathobiology. These techniques routinely interrogate analytes on a genome–wide scale generating comprehensive DKD associated fingerprints. Linking the molecular fingerprints to deep clinical phenotypes may ultimately elucidate the intricate molecular interplay in a disease stage and subtype specific manner. This insight will form the basis for accurate prognosis and facilitate targeted therapeutic interventions. In this review, we present ongoing efforts from large scale data integration translating "–omics" research efforts into improved and individualized health care in DKD.

Keywords

diabetic nephropathy; systems biology; high throughput molecular profiling; transcriptional regulatory networks; proteomics; metabolomics

Introduction

Diabetic kidney disease (DKD) is a common microvascular complication of diabetes mellitus (DM) that is associated with progressive loss of kidney function, systemic endocrine and cardiovascular complications, and premature death. In addition to the personal impact, DKD imposes a great socio–economic burden and constitutes a significant

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Conflict of interest: The authors declare no competing financial interest.

public health challenge. As the epidemic of DM increases worldwide, developing countries will account for ~70% of diabetic patients placing substantial demands on already scarce resources [1,2]. DKD affects roughly 20–40% of individuals suffering from both type 1 (T1DM) and type 2 DM (T2DM) over a 10-year disease period. DKD is therefore not only the single leading cause of incident chronic kidney disease (CKD) but also responsible for over 40% of patients initiating renal replacement therapy due to end stage renal disease (ESRD) in the United States, 18–40% across Europe according to European Registry Data, and 35% and 51% in Australia and New Zealand respectively [3–8]. The increase in DKD induced ESRD is widely attributed to the worldwide growing obesity and T2DM epidemic, accounting for almost 85–90% of DKD cases overall. Furthermore, progressive DKD is an independent risk factor that amplifies morbidity and mortality in patients with diabetes [9–11].

Advances in our understanding of renal physiology and DKD pathogenesis have led to the identification of Renin–Angiotensin–Aldosterone System (RAAS) inhibition, blood glucose and blood pressure control as the pillars of DKD management. These interventions can slow, but usually do not stop progression to ESRD, leaving a large unmet need for novel therapeutic strategies in DKD for more than two decades. Even for established interventions, robust prognostic biomarkers to detect patients at risk for DKD, predicting DKD trajectory and response to therapeutic intervention, are widely lacking. Current classification and treatment strategies primarily rely on a descriptive clinical phenotyping most likely lumping patients with different disease mechanism and stages together. Ongoing research efforts have uncovered potential genetic, behavioral and environmental contributions to disease manifestation in a focused hypothesis driven manner. The development of molecular medicine with genome–wide profiling capabilities of human biosamples offers an opportunity to capture a holistic picture of DKD initiation and progression. Critical questions with this strategy are: How can we effectively exploit these new technologies to facilitate robust and reproducible molecular phenotyping and disease dissection? What proportion of disease depends upon genetic variation? What are the interrelationships between the different puzzle pieces captured by the–omics profiles? How can we integrate this knowledge to define biomarkers of mechanism based disease prediction? How can we distinguish the drivers of pathogenesis from associated, but not causal “passengers” of the disease process? Integrating some of the answers into our current disease understanding and classification will enable us to develop a detailed molecular taxonomy, which identifies specific patient subgroups according to prognostic relevance and necessary therapeutic intervention.

Background

The multifactorial DKD pathogenesis is a consequence of an intricate interplay between genetic factors, environmental exposure and lifestyle. On a molecular level, disease pathogenesis is driven by the hallmark of DM – hyperglycemia, which is caused by incremental lack of insulin in T1DM and target tissue insulin resistance in T2DM [12]. Current understanding suggests that hyperglycemia–driven generation of reactive oxygen species (ROS) and formation of long lasting glycated protein products defined as advanced glycation end products (AGEs) lead to substantial tissue damage. The downstream effects include activation of the intracellular stress response machinery, increased activity of protein kinase C (PKC) pathway, activation of inflammatory signaling mechanisms converging on augmented transcriptional activity of NF–κB, JAK/STAT signaling and a plethora of other effectors [13–15]. Upon initial kidney tissue injury and release of chemoattractants like macrophage chemoattractant MCP–1, infiltrating monocytes/macrophages are a primary source of tumor necrosis factor α (TNF α) release, although resident kidney cells such as endothelial and tubular epithelial cells possess the capability to synthesize this cytokine [16].

TNF–α driven receptor activation changes expression patterns of growth factors, adhesion molecules and other inflammatory molecules. In this hyperglycemic environment TNF–α mediated processes are unleashed from their regulatory constraints, perpetuating an increase of proinflammatory cytokines sustaining further the process of kidney damage. TNF–α has also been implicated in the unfavorable glomerular hemodynamics that characterize DKD e.g. increasing expression of vasoconstrictor endothelin–1, and augmenting glomerular permeability as well as further glomerular and interstitial recruitment of inflammatory cells in DKD [17–22]. DKD associated vascular stress triggers local release of highly vasoactive hormones and peptides influencing activity of the RAAS and endothelin signaling [13]. Activation of the autonomic nervous system [23] can contribute to kidney injury through neuroimmune interaction, modulating interstitial macrophage recruitment and local TNF-α expression during the progressive phase of DKD and CKD in general [24]. Concurrent to the inflammatory processes, there is activation of potent profibrotic mechanisms, which eventually lead to replacement of functional kidney tissue with extracellular matrix, aggravating the progressive phase of DKD. One dominant protagonist is TGF–β (transforming growth factor β) and its downstream mediator CTGF (connective tissue growth factor) [25–27]. This persistent activation of pathogenic mechanisms is not matched by an efficient recruitment of protective pathways (e.g. nitric oxide pathway) leading over time to the specific and well characterized morphological changes and functional deterioration of DKD.

The need for a molecular–based disease definition

The need for early predictors

Diabetic kidney disease develops only in a proportion of patients after decades with DM [8]. Eventually, a subset of those will further progress to ESRD. The current clinical definition of DKD solely relies upon urinary excretion of albumin (usually determined by the urinary **a**lbumin–to–**c**reatinine **r**atio, ACR), using empirically defined thresholds, where ACR < 30 mg/g is termed normalbuminuria (NAlb), 30 ≤ ACR < 300 mg/g defines microalbuminuria (μAlb) and ACR values ≥ 300 mg/g as macroalbuminuria (MAlb) and changes in glomerular filtration rate (GFR) in the presence of T1DM or T2DM. ACR and in particular GFR, are subject to substantial measurement variability, show a heterogeneous pattern and do not necessarily correlate with physiopathology and predict renal function decline [28–31]. Since albuminuria is an early feature of most non–diabetic kidney diseases and can increase with diseases of non–renal origin, it is not very helpful in establishing early diagnosis or in predicting prospective events [32,33]. More than 10 years ago, Caramori and colleagues already emphasized that in context of T1DM GFR changes as well as detection of albuminuria are not biomarkers of early disease stage but rather a sign of significant tissue damage [34]. As such, ACR's and GFR's capability to detect and closely monitor early pathogenic inflammatory and metabolic processes involved in DKD has significant limitations [34–36]. ACR is the predominant clinical "biomarker" for establishing DKD diagnosis and estimating risk of progression based on observational and interventional studies [37]. However, recent analysis from subjects with T1DM enrolled in the Joslin Study of the Natural History of Microalbuminuria suggests that progression of DKD occurs to a substantial proportion even in the absence of μAlb, consequently challenging ACR as a mandatory feature of DKD [35,38–40]. This finding has been corroborated in population–based studies of DKD such as the National Health and Nutrition Examination Study (NHANES) and the Developing Education on Microalbuminuria for Awareness of Renal and Cardiovascular Risk in Diabetes study (DEMAND) [8,41]. Although, the risk of DKD progression is highest among those T1DM individuals with longer diabetes duration, higher HbA_{1c} and increasing albuminuria, early renal function decline strongly supports the concept that renal damage does not begin with or after development of albuminuria stage,

but can occur much earlier. These observations have been corroborated in Native Americans with T2DM–DKD [42]. Additionally, Karalliedde *et al.* pointed out the complex and multifaceted pathophysiology of albuminuria [43]. The phenotype of diabetic albuminuria is further confounded by the substantial ACR variation occasionally creating phenotypic ambiguity [44]. These data clearly show the limits of our current understanding of early changes in DKD pathogenesis and underline the need for research to define the fundamental disease mechanisms and to develop potentially nephroprotective strategies. Furthermore, ethnic differences not only influence the prevalence of DM but also have a substantial impact on the prevalence of diabetes–associated complications and further disease progression. For instance, DKD incidence is lowest in patients of Caucasian descent, followed by Asians, Hispanics, Native Americans and African Americans [45]. On top of racial disparities, DKD susceptibility and rate of progression is clustered in certain pedigrees as results from The Family Investigation of Nephropathy and Diabetes (FIND) study [46,47] and the Genetics of Kidneys in Diabetes (GoKinD) Study showed over the last years [48–54].

Rationale for a systems level definition of DKD

DM and its end organ damage, including DKD, are diseases affecting multiple interrelated end organ systems, requiring a research strategy matching the systemic nature of the disease. Over the last decade both data generation and data analysis approaches have matured to a level that we can start to explore DM and DKD in patients on a systems level, generating a network view of relevant disease processes [55,56]. The term “Systems Biology” in the context of this review is used to describe the integration of large–scale data sets into regulatory networks, using the concept of “Plurality of cause and effect” as introduced by Sauer [57]. Molecular medicine has matured to a level that we are now in a position to generate information from patients covering the entire spectrum from genetic variations such as single nucleotide polymorphisms (SNPs) or copy number variations (CNVs) (***Genomics)***, along epigenetic modifications (methylation and histone modifications; ***Epigenomics***), coding and non-coding RNAs (mRNA, miRNA, lincRNA, tRNA, and rRNA; ***Transcriptomics***), down to the level of proteins (***Proteomics***) and metabolites (***Metabolomics***), which are further refined by detailed morphologic characterization (***Histo–/Morphogenomics***) and in depth ethno–demographics as well as clinical phenotyping (***Phenomics***) along the “genome–phenome continuum” [58–60] (see Figure 1). This approach has already facilitated a more comprehensive understanding of disease pathogenesis in oncology and developmental biology [61]. This is the necessary step towards individualized medicine – “the right drug, at the right time, at the right dose, for the right person” [62] – in nephrology. However, in order to achieve this goal of a tailored and risk adapted therapy we have to implement a systematic approach to define the impact of the disease along the genotype–phenotype continuum [63]. This approach can be broken down in four equally challenging steps:

- **I.** Establish DKD cohorts with deep phenotypes and matching comprehensive biorepositories for genome wide analysis.
- **II.** Map genomic landscape in representative DKD tissue samples (e.g. kidney biopsy, blood and urine).
- **III.** Integrate generated large–scale data sets to define key disease drivers active across multiple levels of the genotype–phenotype continuum.
- **IV.** Test hypotheses derived from III. in model systems and in human interventional trials.

Several DKD cohorts, established over the last two decades, allow capturing of regulatory networks on a genome wide scale, but further investment into patient cohorts as key resources for systems biology are urgently needed. Several strategies are currently discussed to overcome this obstacle ranging from protocol biopsies cohorts to postmortem acquisition of tissue in well phenotyped patients. A recent workshop focused on the discussion of this very key issue pertaining to DKD research [64]. A detailed discussion of this topic warrants an independent review.

Mapping the genomic landscape in DKD

Genetics, Epigenetics and the concept of "metabolic memory" in DKD

Fueled by the discovery of monogenic defects causing rare hereditary forms of diabetes and kidney diseases, strategies were developed to detect the genetic underpinning of DKD. First reports suggesting a genetic contribution to disease risk were based on T1DM sib–pair analysis where DKD subjects aggregated [65,66]. Soon thereafter, reports followed suggesting a heritable component of DKD in T2DM as well [67,68]. Hallmark studies in DKD revealed numerous genetic variations, with some of them replicated across different ethnicities, in T1DM – and T2DM–DKD and some specific for the analyzed cohort and phenotype ([46,69] and Metaanalysis in [70]). The studies published to date identified genetic loci associated with DKD as starting point for functional analysis into disease pathogenesis. However, the overall explained heritability is of low effect size, shared with many other complex traits [71,72]. The very modest genetic effect size confirms the polyetiological nature of the disease. Finding significantly associated genetic variations at a genome wide level for DKD is only the first step. Validation by subsequent resequencing and fine mapping studies to determine the actual causal variant that is in linkage disequilibrium with the tagging SNP, replication in an independent cohort and generation of a testable hypothesis of the mechanistic impact of the polymorphism are critical next steps [73–75]. In oncology, Pomerantz *et al.* recently highlighted such a successful strategy and the importance of experimental follow up [76]. In DKD, the currently available information on genetic risk loci are not of sufficient effect size and robustly replicated as to be developed for clinical decision support, despite the fact that several loci confer a statistically significant increase in disease risk [70,77,78].

Long–term outcome data from DCCT/EDIC and the UKPDS study showing a lasting impact of early intervention on the incidence of diabetic end organ complications [79,80], led to the resurrection of the metabolic memory concept, first described by Engerman and Kern in the dog retina of alloxan–induced diabetes [81]. In addition to long–lasting protein modification like generation of AGEs, epigenetic modifications of the genome are prime candidate memory mechanisms, due to their partial heritability and high plasticity in response to internal and environmental challenges such as drugs, toxins, hyperglycemia and inflammation [82–87]. The area of epigenomics in context of diabetic complications is currently under intense investigation [88–91]. Integrating the epigenetic regulation of the genome with the emerging information of genetic risk loci of DKD might allow defining the individual genetic risk at a clinically relevant level [92]. Rapidly dropping sequencing costs will further facilitate comprehensive sequence based analysis in populations at risk of DKD [93].

Transcriptomics and the next generation profiling of diabetic kidney disease

Transcriptomic analysis, the comprehensive mRNA expression analysis, was the first genome wide profiling approach available for broad application in human tissues [94]. Initially, microarrays using predefined oligonucleotides or cDNAs were employed. These array technologies are currently starting to be replaced by unbiased RNA Sequencing

(RNA–Seq.) technology. Expression profiles can be simultaneously interrogated in kidney tissue, blood and urine, offering the chance to compare across organ boundaries and evaluate their potential for non–invasive surrogate marker. With the adoption of a standardized technique of specimen handling and RNA isolation developed by the European Renal cDNA Bank, a robust protocol was established taking advantage of diagnostic renal biopsies for molecular profiling studies in an international multicenter setting [95,96] and the approach has been adopted by multiple networks around the globe. Initial studies focused on targeted analysis of candidate transcripts identified by the analysis of monogenetic renal diseases [97] or model systems [98], followed by studies comparing *in vitro* responses to proteinuria to the *in vivo* setting [99]. Baelde and colleagues compared the glomerular transcriptome from two cadaveric healthy donors with two DKD cases and where able to partially recapitulate findings from prior studies investigating gene expression changes in cell culture, animal models and human biopsy sections [100]. A first comprehensive transcriptional network analysis combined with promoter modeling approaches identified a specific NF–κB model as a key driver in progressive DKD compared to early DKD and other proteinuric diseases [101]. Additionally, the mechanism involving hypoxic gene regulation, vascular plasticity as well as JAK/STAT signaling pathway show temporal and compartment specific gene expression patterns in human DKD [15,102]. Furthermore, transcriptomic profiling not only validated prior documented significance of complement pathway and inflammatory signaling pathways but also described new pathways involved and/or affected by DKD, which haven't been suspected yet or underestimated e.g. caveolar–mediated endocytosis and lipid metabolism [103]. Current limitations of kidney tissue specific transcriptomics relate primarily to the limited available sample size and the cross sectional design with limited inference of causality. Most studies using clinical biopsies represent established DKD stages with impaired eGFR and high degree of proteinuria and describe the inflammatory and fibrotic response seen with progression to ESRD [101,103]. A significant concern is the selection bias for patients with suspected DKD undergoing biopsy, as kidney biopsy in most centers is not part of the routine DKD diagnostic evaluation, except in cases of sudden unexplained rise in serum creatinine, drop in eGFR or increased proteinuria [104], requiring careful evaluation of clinical and histological features to select cases of DKD without secondary disease manifestation.

Several groups have implemented renal research or protocol biopsies in early stages of T1DM– and T2DM–DKD [105,106]. A low incidence in kidney biopsy related complications in carefully selected patients and improved diagnostic value for disease entities potentially amenable to treatment have been reported with modern biopsy technologies [107,108]. The unique renal macro– and microarchitecture with multiple cell lineages arranged in a defined order and spacing along the nephron (e.g. podocytes, mesangial cells) contribute to substantial heterogeneity. This can be addressed to some extent by manual microdissection of the nephron compartments [109,110] or "in silico" microdissection using cell–lineage specific transcripts [97]. Methods using the concept of cell lineage specific transcripts on a genome scale to deconvolve the transcriptome of complex tissues are currently being developed [111].

As with all genome wide profiling approaches, validating diagnostic candidate markers of molecular pathways in an independent replication cohort is a critical step to address the inherent danger of overfitting large–scale data on small sample sizes (see [112] and a detailed discussion by the Institute of Medicine [113]). Generating the necessary cohorts and subsequent data analysis will require further integration of existing efforts and expansion of international collaborative networks. As most groups use comparable tissue procurement, processing and analysis platforms for transcriptional profiling, data integration and comparative analysis are an achievable goal.

Proteomics and Metabolomics in DKD

Advances in high throughput profiling technologies facilitated an unprecedented explosion in generating large–scale molecular data that is not any longer constrained to the genome and transcriptome level, but also leads to significant improvements on the level of proteomics and metabolomics. The proteomic studies start to highlight the substantial role of posttranscriptional regulatory mechanisms and simultaneously reveal how gene expression affects actual protein abundance and function [114–116]. The various layers of posttranscriptional and posttranslational regulation add an additional level of complexity in proteomic studies. Major advances in analytical chemistry, including improved chromatographic and mass spectrometry (MS) platforms supported by sophisticated computational algorithms are finally starting to bring proteomics towards a level of protein coverage for widespread use in systems biology [117,118].

Proteomics

Since ACR does not accurately predict clinical endpoints in DKD, several investigators have focused on urinary and plasma proteome patterns from healthy volunteers, T1DM and T2DM patients with and without various degrees of albuminuria and different ranges of GFR [119–125]. Three recent examples highlight the potential of an untargeted proteomics approach. Otu and colleagues using a nested case–control design and surface–enhanced laser desorption/ionization time–of–flight mass spectrometry (SELDI–TOF/MS) of urine from type 2 DM Pima Indians were able to distinguish initially NAlb subjects developing DKD within a 10 year follow up period based on a 12–peak urine peptide signature detected in urine collected at time the individuals were still NAlb [126]. Rossing and co–workers compared gender and age matched healthy controls, and T1DM patients from the Steno Diabetes Center with varying degrees of albuminuria (NAlb, μAlb and MAlb) [124]. They evaluated the potential of Capillary electrophoresis (CE) MS to define urinary polypeptides signatures an identified increased albumin fragments, decreased uromodulin and collagen type I fragments. Overgaard *et al*. utilized isobaric mass tags (iTRAQ) coupled with liquid chromatography (LC) MS and SELDI–TOF/MS to identify plasma protein/peptides in a cross–sectional analysis of 123 T1DM cases previously classified as NAlb, μAlb and MAlb. This strategy yielded many promising candidates including transthyretin, apolipoprotein A1, apolipoprotein C1 and cystatin c. Interestingly, unidentified plasma protein peaks showed promising features in improving predictability of DKD [127,128]. Other studies applied CE–MS and confirmed urinary peptides such as collagen fragments as robust predictors of CKD and DKD respectively [129–133]. One of the limitations of these studies is that majority of the identified fragments arise from abundant plasma proteins. As the glomerular filtration barrier fails with DKD, non–selective, albumin predominant pattern ensues which may skew detected polypeptides to abundant proteins [134]. Methods effectively addressing the detection of low abundance proteins have recently been developed, in order to provide a higher degree of disease specificity and unravel low–abundant proteins which are likely to be of mechanistic significance [135]. Immunodepletion and glycoprotein enrichment techniques allow effective depletion of albumin, immunoglobulins and other highly abundant urinary and plasma proteins while providing a more in depth analysis of a sub fraction of the inquired proteome. As glycosylated proteins are critical for cellular interactions and signaling cascades, disease states are likely to cause early and specific alterations in urinary glycoprotein excretion. Indeed, glycoproteins are now important markers of autoimmunity and malignancy [136,137]. More recently, the plasma glycoproteome has been used to predict nephropathy in diabetic subjects [138]. Despite this promising role as a non–invasive and specific biomarker of disease, little is known about the urinary glycoproteome in DKD. Consequently, we hypothesized that the urinary glycoproteome would be altered in patients with CKD compared to healthy controls, and that specific glycoprotein alterations might be useful in predicting CKD progression. We

performed an exploratory analysis of the urine glycoproteins in patients with CKD that included DKD subjects [139]. We utilized a hydrazide enrichment technique combined with tandem MS identification of glycoproteins. In urine, this strategy identified several differentially expressed proteins compared to healthy controls, including proteins with endopeptidase inhibitor activity, protein–binding functions, and acute–phase/immune–stress response activity that have been previously linked to kidney disease and therefore supporting the existing hypotheses that inflammation plays a key role in renal disease pathogenesis. Additional renal specific sources of proteins include analysis of excreted urinary exosomes (30–90 nm vesicles), which consist of an evolutionary conserved tissue– and cell–type specific set of proteins. Due to its tissue specificity, exosomes analysis might facilitate a more accurate representation of renal pathology [140,141]. Despite these advances, several challenges remain in proteomic analysis. These include: **a)** impaired detection of low–abundant proteins and skewing to high–abundant proteins especially in blood and urine (while strategies involving depletion of high–abundant proteins and enrichment of low–abundant proteins have improved detection, coverage still lacks depth); **b)** obstacles dependent on employed techniques and software data analysis issues [142] and **c)** difficulty in predicting the functional context of the protein. For example protein abundance may vary but functionality may not change as it may depend on posttranslational modifications such phosphorylation, acetylation and glycosylation. These issues have been recently addressed in a position statement including recommendations for quality and reporting metrics, data deposition and quantification methods [143–145], similar to the MIAME reporting guidelines for transcriptomic analysis [146]. Collectively, these observations suggest that the human proteome and in particular the glycoproteome may serve as a discovery source for novel disease mechanism–based biomarkers not only in DKD but also in renal diseases in general but challenges remain in translating these findings to functional context.

Metabolomics

Metabolomics is the rapidly evolving field that attempts to systematically capture, identify and quantify endogenous and exogenous metabolites within a biological sample. The small molecules represent the result of complex biological processes in a given tissue or organ and the final functional read–out of a biological system. Therefore, metabolites form attractive candidates as biomarkers, as they are closer to the final phenotype. The chemical diversity of the metabolites makes analysis a formidable challenge. The rapid development of a variety of MS and nuclear magnetic resonance (NMR) based platforms have enabled detection, separation, characterization and quantification of such chemically diverse low–molecular weight structures such as lipids, amino acids, nucleic acids and organic acids (reviewed in [118]). Metabolite profiling strategies have been implemented successfully to identify metabolic markers of disease in cancer, cardiovascular disease (CVD) and diabetes [147–155]. We recently utilized an untargeted metabolomic strategy to explore underlying unifying mechanisms of disease progression and treatment response in order to identify novel metabolite biomarkers of progressive murine DKD [156]. The urine from control DBA/2J and streptozotocin–induced diabetic mice as well as mice treated for 10 weeks with rosiglitazone (which reverses DKD phenotype) was analyzed with LC electrospray ionization (ESI) TOF/MS [156]. In total 56 features showed up– or down–regulation by > 2–fold in the diabetic animals. Of the 56 molecular features, 32 were identified by database searching. Rosiglitazone reversed nine of these 32 compounds back to baseline and may therefore serve as potential biomarkers for response to treatment and reversal of rodent DKD phenotype. For compound identification, we leveraged the publicly available METLIN Metabolite Database [157] and the Human Metabolome Database [158]. The pathophysiology of the metabolic changes that include ubiquinone and indoxyl sulfate is currently under intense investigation. Interestingly, a study from Barreto *et al.* reported that

serum indoxyl sulfate correlates inversely with renal function and might have a direct relationship with aortic calcification and pulse wave velocity in patients with CKD [159]. In survival analyses, the highest indoxyl sulfate tertile was a powerful predictor of overall and cardiovascular mortality. The predictive power of indoxyl sulfate for death was retained after adjustment for age, gender, diabetes, albumin, hemoglobin, phosphate and aortic calcification. Other clinical studies highlight the potential application of metabolomics in DKD research. The study from Zhang and colleagues utilized Ultra performance liquid chromatography (UPLC) coupled with TOF/MS to distinguish the serum metabolic profiles of 25 healthy volunteers, 33 T2DM and 8 DKD patients [160]. Significant changes in the serum level of leucine, dihydrosphingosine and phytosphingosine were noted, indicating perturbations of amino acid metabolism and phospholipid metabolism. Another study investigated 52 T1DM patients recruited in the FinnDiane Cohort who were prospectively followed over 5–6 years, during which one half developed proteinuria (progressors) and the other half did not (non–progressor) [161]. Metabolite profiles of baseline 24–h urine samples were analyzed by gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS). Multivariate logistic regression modeling of the GC MS data resulted in a classifier profile that had an overall accuracy of 75% and a precision of 73% for a binary classification model of progressor vs. non–progressor. The discriminating metabolites included acyl–carnitines, acyl–glycines, and metabolites related to tryptophan metabolism. Other studies of DKD yielded metabolic profiles indicating alterations in carbohydrate, lipid, nucleic acid, and amino acid metabolism [121,162]. The biomarker potential for these metabolic markers for predicting clinical phenotypes need to be validated in large–scale clinical trials.

Systems Genetics: linking genetic variance to clinical outcomes via intermediary phenotypes

Genome wide association studies (GWAS) of complex traits have been very effective in defining statistically significant risk loci by mapping genome wide genetic variance in large cohorts onto clinical traits. However, this approach is agnostic concerning the molecular mechanism and intermediate regulatory cascades responsible for the manifestation of the clinical phenotype. To close the molecular knowledge gap, systems genetics employs as a key tool association studies linking genotypic information with intermediary molecular traits of interest, e.g. transcript level or metabolite level (**e**xpression/**m**etabolic **Q**uantitative **T**rait **L**ocus, eQTL/mQTL) [163–165]. Subsequent integration of molecular QTLs with association studies aids to identify the molecular impact of genetic variance associated with a disease phenotype.

For instance, the metabolome, as a close “proxy” of the final phenotype in metabolic diseases like DM, can be associated with the genetic variance seen in a population to establish links between genetic variance and metabolite levels in a defined biosample during a disease process [166–169]. A key study by Ferrara *et al*. linked tissue metabolite levels to genetic variation. The authors performed transcriptome analysis and whole genome sequencing of liver samples from a diabetes–resistant C57BL/6 *leptin*$^{ob/ob}$ and diabetes–susceptible BTBR *leptin*$^{ob/ob}$ mouse strain and further refined their analysis by integrating quantitative metabolite levels [168]. Various groups of liver metabolites significantly associated with distinct chromosomal regions suggesting that they were under the control of genetic variations in those regions. Suhre *et al*. reported on the genetic association of urinary metabolites detected by NMR spectroscopy in urine of 862 male participants from the epidemiological Study of Health in Pomerania (SHIP) [170]. An independent validation in additional 2,031 samples (1,039 independent SHIP and 992 samples from the Cooperative Health Research in the Augsburg Region (KORA) study) revealed consistent genome wide

significant loci tagging SLC7A9 and NAT2 which have been already associated with CKD and drug–induced liver toxicity respectively [171–173]. Kettunen *et al.* utilized NMR spectroscopy based detection of serum metabolites of over 8,000 genotyped Finish individuals and were able to ascertain a high degree of heritability for metabolic phenotypes, ranging from 40% up to 60% [174]. The quantitative traits of various body fluids are currently being mapped for intra– and inter–individual comparison on a population scale [175,176]. While these represent early stage discoveries, they already help to bring genetic variance closer to disease phenotypes.

Building regulatory networks in health and disease

Networks are increasingly used to display complex dependencies in biological systems. A network is a graph consisting of nodes and edges. Nodes represent specific entities or objects for instance a gene, a protein or trait of interest and the edges represent a defined relationship for connected nodes (e.g. protein–protein interaction). These relationships can be directed, where node A interacts and activates node B, or without a specific directionality merely describing an interaction. As each level of information has its own merits and weaknesses, the integration of multiple –omics layers might circumvent or minimize those drawbacks and deliver a more accurate picture of the ongoing processes [177–181]. One such way is to generate and visualize integrated, functional networks by combining genomic information, transcriptomic and proteomic data obtained from the same individual tissue. This approach aids in understanding how DNA sequence variation (e.g. from GWAS) and transcriptional events lead to corresponding changes in pathway functionality. A key aspect of network Employing such a strategy enables the characterization of genetically determined transcriptional regulatory networks in health and their perturbation in disease [101,182]. Moreover, by combining results from time series experiments one can expand and track dynamic changes within those networks over time and detect driving core components and even distinguish between early or late events in the disease process [183,184]. Leveraging this uniform integrative strategy allows combining the strengths and compensates for the limitations on each of the multiple lines of evidence. The resulting hierarchical and comprehensive “disease map”, defines key molecular functionalities, highlighting points of conversion that might be more amenable for further exploration and therapeutic intervention [185]. Through a combination of causal and probabilistic methods across mouse and humans, Schadt and co–workers were able to pin point key functionalities with high confidence and provide a framework for targeted downstream experimental evaluation. Due to the unbiased nature of these strategies, integrative approaches are capable of uncovering new disease specific relationships and can shed additional light into cellular and organismal pathophysiology [184,186,187].

A crucial step in advancing integrative network biology analysis into the clinical arena will rely on a robust and comprehensive visualization of these multilayered interactions [188,189]. Cytoscape, the versatile open source bioinformatics platform is the most common and powerful tool used to visualize and analyze networks [190]. Even though network representation has proven useful in the past years, networks are still compressing multidimensional data sets into two or maximal three dimensions [191] and represent therefore a highly simplified version of the true underlying dynamic processes. This “static” network concept has to be expanded to capture dynamic interactions and to adapt to environmental and genetic constraints over time in order to gain full insight into cellular dynamics [184]. Defining human health and disease on a genome wide scale has already yielded very exciting results across the genome–phenome continuum. The concept of integrated personal –omics profiling (iPOP) is increasingly gaining attraction [192], but challenges remain: ***a)*** discovery of rare variants and variants with smaller effect sizes, ***b)***

understanding the link between genetic variations and perturbation of pathophysiology, and finally ***c)*** translating –omics results efficiently into improved patient care.

Biomarker: the surrogates for diagnosis, risk assessment and prognosis

In order to facilitate an accurate diagnosis, predict risk of progression and response to drug/dietary intervention clinical medicine relies on specific, disease–associated biomarkers. Biomarkers are objective, quantitatively or qualitatively measured characteristics indicating normal or pathogenic biological processes or response to therapeutic intervention [193–195]. Sensitivity, specificity and positive or negative predictive value of those markers influence our therapeutic decision–making and patient counseling. However, the available biomarkers for DKD, such as ACR and GFR, harbor significant limitations and can be misleading in the research and clinical setting (see above). The optimal biomarker should reflect consistency, strength of relationship between “marker” and outcome in question, i.e. for DKD progression to ESRD. Biomarkers strength are of higher impact if an adequate characterization of disease dynamics can be established *a priori* e.g. the marker precedes the desired outcome, outperforms the already established disease predictors and responds favorably to therapeutic interventions that are known to have salutary effects on disease progression. The degree of confidence can be augmented further if the biomarker does not only show a pure “guilt by association” characteristic but is also an integral part of the pathophysiologic process in question [43,196]. Biomarkers can be classified according to their purpose: diagnostic, disease staging and prognostic, as well as source based – genomic, transcriptomic, proteomic and metabolomic marker(s). The field of biomarkers in DKD and kidney disease in general is rapidly evolving and has been extensively reviewed in [119,197,198].

Challenges and perspectives

What we can learn from Oncology

Over the past years, we witnessed a substantial progress in uncovering new pathophysiologic mechanisms of neoplastic disease and their explicit characterization on a molecular and network level. The advent of profiling technology enabled the transition from a pure experimental screening method to implementation of tumor specific fingerprinting in prospective clinical trials guiding therapeutic decision [199]. However, recent studies showed that vigorous analytical assessment of generated data as well as documentation of robust reproducibility in independent cohorts is of paramount importance, as ambiguous results can impact the decision making process leading to misassignment of targeted therapeutics and severely harm patients [200,201]. One such instance that served as a wakeup call for the entire –omics research community is the experience from a recent case involving scientific misconduct in translating –omics research results to clinical patient stratification without rigorous validation [202,203]. After retraction of a series of articles in Journal of Clinical Oncology, Nature Medicine and New England Journal of Medicine [204–206], and at request of the National Cancer Institute (NCI) the recently released report from the Institute of Medicine (IOM) pertaining to the topic of “translational OMICS” establishes guidelines to assure reproducible omics–based test technology grounded on sound scientific conduct [113]. Therefore, robust validation strategies need to be employed, including oversight by federal regulatory agencies such as the Food and Drug Administration (FDA), when the intended use of developed tests will extend into clinical trials [207].

Thus far, the limited studies using a data mining strategy on genome wide DKD data sets have not only confirmed established pathophysiological mechanism, but also described novel mechanisms associated with disease pathogenesis. One major aspect contributing to the limited capability of robust mechanistic discoveries and uncovering potential

confounding factors is the availability of high quality samples for –omics analysis in conjunction with in–depth clinical phenotyping data in sufficiently sized cohorts. However, increasing the sample size is and can only be a first step in the process of dissecting the underlying molecular mechanisms of DKD. Curtis *et al.* demonstrated the power of integrative –omics data analysis in combination with longitudinal follow up of over 2,000 clinically annotated breast cancer specimens [208]. Based on experimental, observational and interventional data DKD can be considered as a spectrum of "diseases" with extreme phenotypes at each end e.g. fast progressors vs. non–progressors regardless of intervention ("resistant to the effect of poor metabolic control" [209]). The Joslin 50–year Medalist Study has established a longstanding cohort of T1DM patients with no apparent end organ damage after decades of DM [210]. The hope is that these cohorts will allow to capture the genetic and environmental sources of phenotypic heterogeneity in DKD.

An additional source of mechanistic heterogeneity seen in DKD relates to disease process activation in a stage specific manner. Pathogenic disease processes such as ROS generation, inflammation and profibrotic pathway activation may contribute differently at various disease stages and might not be equally affected by therapeutic interventions. Loss of differentiation and inflammation might play a more significant role during initiation of complications, but might be superseded in late stages of progression by profibrotic mechanisms. To distinguish these events sufficient large cohorts with clinical and structural information on disease stage are essential. However, even modest samples size can allow to define a compartment and stage–specific regulation, as has been demonstrated for the JAK/STAT pathway with activation during early DKD in the glomerular compartment, followed by the tubulointerstitial compartment during the progressive phase of the disease [15].

Tackling critical steps in a collaborative effort in order to assemble necessary cohorts for multilevel outcome analysis is a crucial challenge in DKD. These strategies are already implemented in other glomerular diseases i.e. as realized for Nephrotic Syndrome with the **Nep**hrotic Syndrome S**tu**dy **Ne**twork (NEPTUNE, [211]) where a multidisciplinary team of scientists work together with patients and their families towards a comprehensive picture of glomerular failure. To ensure effective use of large–scale data sets by the renal research community requires innovative concepts of data processing and sharing between different knowledge domains (for a formal analysis of this collaborative interdisciplinary process see [212]). We need easily accessible platforms that allow data storage in reasonable accessible repositories, facilitate data exchange between involved researchers, and allow dissemination to the research community. First tools serving these functionalities in defined knowledge domains are emerging (i.e. Nephromine for renal transcriptional data sets [213]) and more comprehensive solutions concerning this key challenge of large–scale data research are currently pursued [214].

Concluding remarks

DKD develops as a consequence of systemic disease, diabetes, driving the intrarenal disease progression via a multitude of interrelated molecular pathways. DKD and other diabetic complications, particularly CVD, interact via multiple feedback loops leading to the demise of the entire organism. Molecular medicine strategies described in this review allow for the first time to capture the complexity of these individual regulatory events in a genome wide manner. The emerging tools for network–based analysis of biological systems will provide a starting point to understand the regulatory cascades of DKD on a tissue and organismal level. Matching the complexity of DKD disease process with a comprehensive research approach should allow defining the key drivers across disease stages and organs. This will define the critical entry points for molecular based diagnostics, prognostics and targeted therapy.

Acknowledgments

This work was supported by grants from the Juvenile Diabetes Research Foundation to M.K. and S.P., the German Research Foundation to C.V.K. (KO 4266/1-1), the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease (P30-DK-081943 to F.C.B. and M.K, DK083912 to M.K., DK089503, DK094292 to S.P., DK082841 to M.K. and S.P.). The authors appreciate the assistance of Dr. Jeff B. Hodgin (Department of Pathology, University of Michigan) for providing the histological images. We further thank Chandra Hall for carefully proofreading the manuscript and the editor and referees for helpful comments and suggestions on an earlier version of the manuscript. The authors apologize to those colleagues whose work we were unable to cite in this review due to space limitation.

Abbreviations

GWAS	genome wide association study
SNP	single nucleotide polymorphism
CNV	copy number variation
NF–κB	nuclear factor κ–light–chain–enhancer of activated B cells
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
MCP–1/CCL2	macrophage chemoattractant protein 1
FDA	U.S. Food and Drug Administration
DCCT/EDIC	The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study
UKPDS	The United Kingdom Prospective Diabetes Study
ERCB	European Renal cDNA Bank–Kröner–Fresenius Biospy Bank
MIAME	Minimum Information About a Microarray Experiment

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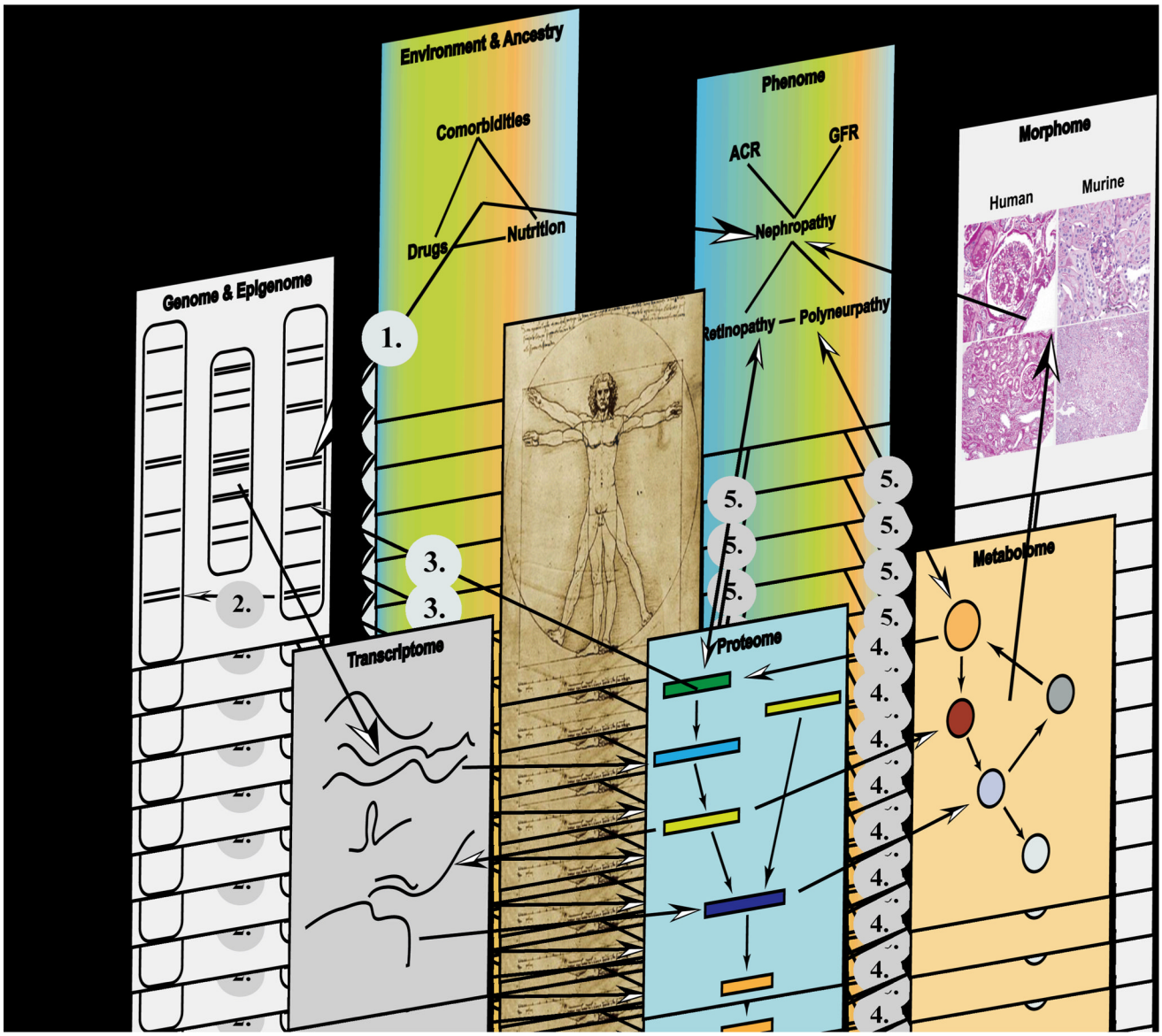

Figure 1. From Genome–to–Phenome – An Integrative Systems Biology Perspective on Diabetic Kidney Disease

Each biological system (e.g. diabetic kidney) can be regarded as being composed of discrete components. Each component can be independently inquired through unbiased high throughput "–omics" profiling or imaging technologies (ultrasound, nuclear magnetic resonance imaging, positron emission tomography), but individual level data is embedded in an interaction network that connects and integrates the various components in a functional context. Potential interactions between two or more components are indicated through arrows e.g. gene–by–environment interaction (***1***), gene–by–gene interaction (***2***), protein–DNA interaction (***3***), metabolite–protein interaction (***4***) e.g. allosteric regulation of enzymes by small metabolites and reciprocal Phenome–"omes" interaction (***5***). Technologies applied to generate –omics level data are: **Genome/Epigenome** (SNPs, CNV, promoters): e.g. genome sequencing, high–density SNP chips, methylation assays (MeDIP), histone modifications; **Transcriptome** (mRNA, miRNA, lncRNA, piRNA, tRNA, rRNA):

microarray(s) and RNA sequencing; **Proteome** (Phosphoproteome, Glycoproteome, Acetylome): yeast two–hybrid, coimmunoprecipitation, 2D differential gel electrophoresis; **Metabolome** (sugars, lipids, amino acids, small molecules): nuclear magnetic resonance spectroscopy and mass spectrometry; **Morphome** (tissue damage/integrity): histology, immunohistochemistry; **Phenome** (ACR, GFR, retinal microaneurysm): clinical chemistry, clearance measurement, imaging studies; **Environment and Ancestry** (Drugs, Nutrition, Comorbidities): nutritional assessment, environmental exposure and socio–economic assessment.