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REVIEW ARTICLE

Novel biomarkers of diabetic kidney disease: current status and potential clinical application

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Abstract

Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease (ESRD). Although both albuminuria and glomerular filtration rate (GFR) are well-established diagnostic/prognostic biomarkers of DKD, they have important limitations. There is, thus, increasing quest to find novel biomarkers to identify the disease in an early stage and to improve risk stratification. In this review, we will outline the major pitfalls of currently available markers, describe promising novel biomarkers, and discuss their potential clinical relevance. In particular, we will focus on the importance of recent advancements in multi-omic technologies in the discovery of new DKD biomarkers. In addition, we will provide an update on new emerging approaches to explore renal function and structure, using functional tests and imaging.

Keywords Diabetic kidney disease · Albuminuria · Omics · Biomarkers · Glomerular filtration rate

Introduction

Diabetic kidney disease (DKD) is a long-term diabetes complication, affecting approximately 30% of patients with type 1 diabetes (T1DM) and 40% of those with type 2 diabetes (T2DM) [1]. DKD is a leading cause of ESRD worldwide, accounting for approximately 40% of new patients requiring renal replacement therapy (RRT). Furthermore, even early stages of DKD confer a substantial increase in the risk of cardiovascular diseases (CVD).

Albuminuria and estimated glomerular filtration rate (eGFR) are the diagnostic/prognostic biomarkers of DKD currently used in clinical practise. However, a substantial proportion of T2DM patients with DKD are normoalbuminuric (non-albuminuric phenotype) and eGFR is the only available biomarker in this subgroup [2].

Both albuminuria and eGFR loss are non-specific markers of DKD, as they are altered in most chronic glomerulopathies. In kidney biopsy studies, a high percentage of T2DM patients do not have diabetic nephropathy, but other kidney diseases or mixed forms [3], though these studies greatly overestimate non-diabetic renal disease as biopsies are usually performed for clinical purposes in patients with a high suspicion of other kidney diseases [4–6]. In addition, comorbidities of T2DM, such obesity, hypertension, and vascular disease, may also contribute to eGFR decline in diabetes. Therefore, DKD can be considered as an umbrella term that includes other renal diseases, intermediate forms, and associated conditions favouring progression (Fig. 1). This heterogeneity is also found in patients recruited in randomized clinical intervention trials (RCT) and represents an important limit to clinical research in the field, as the efficacy of a drug in patients with diabetic nephropathy will be diluted by the presence of the other subgroups.

Both albuminuria and eGFR have also important limits as prognostic tools. Indeed, patients with microalbuminuria not only can progress to macroalbuminuria, but also regress to normoalbuminuria [7]. Moreover, eGFR does not accurately reflect the severity of the kidney damage and when eGFR reaches the threshold of 60 ml/min/1.73m^2 almost 60% of the nephrons are already lost. Indeed, GFR is the product of the number of nephrons times the mean single nephron glomerular filtration rate (SN-GFR) and a reduction in the number of nephrons due to kidney damage can be compensated by an increase in SN-GFR of surviving nephrons (renal functional reserve). It is only when all remaining nephrons

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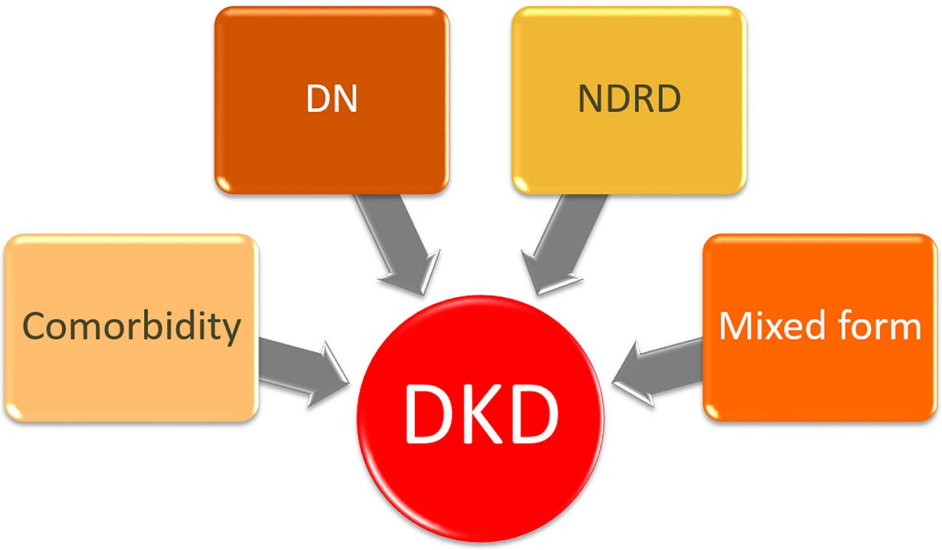

Fig. 1. Diabetic kidney disease (DKD) may be considered an umbrella term including diabetic nephropathy (DN), non-diabetic renal diseases (NDRD), intermediate forms (DN & NDRD), and conditions associated with T2DM, such as hypertension, obesity, vascular diseases that can contribute to kidney damage and favour disease progression

reach their maximal filtration capacity that a further nephron loss results in eGFR decline and the relationship between eGFR and renal damage becomes linear [8,9]. To further complicate the matter, vasodilation of the afferent arteriole in diabetes can increase SN-GFR even in the absence of nephron loss [10]. This has important prognostic implications as a patient without renal damage and a patient, who has already consumed the whole renal functional reserve to compensate the renal damage, can have an identical eGFR, but their prognosis is dramatically different (Fig. 2).

Given the limitations of current markers, there is the need to identify novel diagnostic/prognostic biomarkers for DKD. Herein, we will summarise available data on novel candidate biomarkers and discuss their potential clinical relevance. Moreover, we will review new emerging approaches to biomarker discovery using multi-omic technologies, functional tests, and imaging techniques.

Candidate biomarkers

Candidate biomarkers play a role in inflammation, fibrosis, endothelial dysfunction, tubular injury and they have been selected on the basis of in vitro and in vivo studies that suggested their involvement in the pathogenesis of DKD. The number of serum/urine molecules that have been proposed as candidate biomarkers of DKD is very large. However, it diminishes substantially if we only consider biomarkers that were found associated with relevant DKD outcomes in large longitudinal studies, independently of confounders and risk factors, including both albumin excretion rate (AER) and eGFR. In this section, we will review available data on the most promising biomarkers. Details of the studies described are reported in Table 1.

TNF-α receptors (TNFRs)

TNF-α is an inflammatory cytokine implicated in both the pathogenesis and progression of DKD. TNF-α binds to type 1 (TNFR1) and type 2 (TNFR2) TNF-α receptors. Both receptors are also found in the circulation as soluble forms. Longitudinal studies provided convincing evidence that circulating TNFR levels, particularly TNFR1, are excellent predictors of ESRD in both Caucasians and American Pima Indians patients with T2DM with and without proteinuria [11,12]. Importantly, this was independent of confounders and known risk factors, including glycated hemoglobin (HbA1c), AER and eGFR. Moreover, the ability of TNFRs to predict progression was specific of DKD, as it was not observed in other kidney diseases [13].

Similar results were also obtained in T1DM with macroalbuminuria. In the FinnDiane cohort, TNFR1 was independently associated with the cumulative incidence of ESRD [14] and in the Joslin cohort TNFR2 was the strongest determinant of eGFR decline and time to ESRD onset [15].

Besides predicting the risk of progression, TNFRs are also important in identifying patients who are at high risk of future DKD onset. Indeed, in T1DM patients with normo/microalbuminuria and normal renal function, high TNFR levels predicted a fast early eGFR decline ($\geq$ 3.3%/year) and they were strongly associated with the risk of incident stage 3 CKD (CKD-3) [16,17]. Consistent with the notion that TNFRs are relevant biomarker also in an early stage of DKD, TNFR levels were significantly and independently associated with early glomerular structural lesions (reduced percentage of normally fenestrated endothelium and increased mesangial fractional volume) [18].

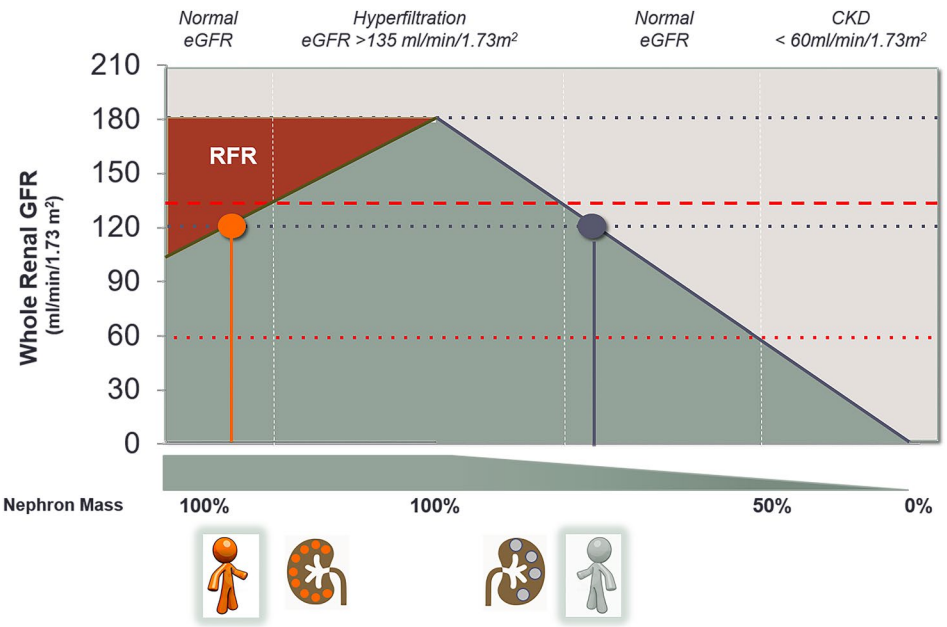

Fig. 2. GFR course during the natural history of DKD. The brown triangle depicts the renal functional reserve (RFR). The red horizontal lines show GFR threshold levels for both whole kidney hyperfiltration and CKD stage 3 definition (>135 and <60 ml/min/1.73m^2 respectively). In an early stage of DKD, increased glomerular capillary hypertension may result in both single nephron and whole kidney hyperfiltration with consumption of the RFR (hyperfiltration stage). Despite significant loss in nephron mass whole kidney GFR may remain normal (normal filtration stage with nephron loss), but rapidly declines towards CKD stage 3. The two subjects represented below the graph have an identical and normal GFR (120 ml/min/1.73m^2); however, the orange subject is in a very early stage of DKD and has normal number of nephrons (depicted as orange circles within the kidney), while the grey subject has a significant reduction in the number of nephrons, but maintains a normal whole-kidney GFR by increasing single nephron-GFR (enlarged grey circles within the kidney). On prognostic viewpoint the two subjects are very different, as the grey individual will rapidly progress towards stage 3 CKD. *Adapted from Tonneijck et al. JASN 2017, 28:1023-1039*

Recently, a study on patients enrolled in the CANTATA-SU trial showed that treatment with the SGLT2 inhibitor (SGLT2i) canaglifozin reduced circulating TNFR1 levels in a dose-dependent manner and that this effect was independently associated with a lesser degree of eGFR decline. Therefore, TNFR1 may also be proposed as biomarker of response to treatment [19]. Finally, measurement of TNFR1 can be used in the recruitment phase of RCT to identify patients at high-risk of progression [20].

Uric acid

Ten years ago two prospective studies in T1DM patients showed that baseline serum uric acid (UA) predicted the development of micro/macroalbuminuria at follow-up [21,22]. Subsequent studies proved that UA levels also predicted a fast early eGFR decline leading to CKD-3 onset in T1DM patients with normal renal function [23,24]. A large real-life epidemiological study confirmed this finding in normoalbuminuric T2DM patients [25]. Moreover, UA-predicted CKD-3 onset also in patients who remained normoalbuminuric at follow-up, suggesting that UA may be a biomarker in patients with the non-albuminuric phenotype of DKD [25].

More recent studies explored whether UA is a predictor of DKD progression in T1DM. In the FinnDiane, cohort baseline UA levels were independently associated with progression towards advanced CKD (stage 4–5) [26]. Consistent with this, in the Steno, cohort UA was an independent predictor of eGFR decline ≥30%, cardiovascular events, and mortality [27].

Taken together these data indicate that UA is a promising biomarker of both early and advanced DKD. This together with basic science evidence of deleterious effects of UA on the kidney led to the controversial hypothesis that UA may also be a potential target for treatment. However, a recent RCT showed that lowering UA level with allopurinol does not slow GFR decline in T1DM with early-to-moderate DKD [28]; therefore, there is no scientific evidence to support the therapeutic use of allopurinol in patients with DKD.

Copeptin

Copeptin, a surrogate marker for arginine vasopressin, was found independently associated with progression to ESRD

Table 1 Main studies on biomarkers and DKD

Biomarker	Type of DM	*n*	Baseline Characteristics	FU (yrs)	Biofluid	Outcomes	Main Results HR (95% CI),	Adjustments	Ref
TNFR1/R2	T2	410	eGFR > 30 – N, Mi, Ma	12	Plasma	Progression to ESRD	TNFR1: HR 4.7 (1.3–17.0)	HbA1c, AER, eGFR	[11]
							TNFR2: HR 2.2 (0.7–6.4)		
TNFR1/R2	T2	193	m-GFR ≥ 60 (89%) -	9.5	Serum	Progression to ESRD	TNFR1: HR 1.6 (1.1–2.2)	Age, gender, HbA1c, BP, m-GFR, ACR	[12]
			N, Mi, Ma				TNFR2: HR 1.7 (1.2–2.3)		
TNFR2	T1	349	eGFR > 30 Ma	7	Serum	Time to ESRD	-34.6% (-39.3,-29.8)	HbA1c, AER, eGFR	[15]
TNFR1	T1	429	Ma	9.4	Plasma	Progression to ESRD	HR: 0.01 (0.00–0.01)*	eGFR, HbA1c, T1DM duration	[14]
TNFR1	T2	194 T2	eGFR > 15	3	Serum	≥ 30% eGFR decline, RRT, renal death	T2DM: 3.8 (1.1–12.8)	Age, sex, eGFR, ACR	[13]
	No-DM	259 No-DM					No-DM: 0.2 (0.03–1.0)		
TNFR1/R2	T1	628	eGFR > 60 – N and Mi	12	Serum	Stage 3 CKD	TNFR1: 2.5 (1.4–4.7)	HbA1c, AER, eGFR,	[17]
							TNFR2: 3.0 (1.7–5.5)		
TNFR1/R2	T1	534	eGFR > 60—N and Mi	8	Serum	eGFR loss ≥ 3.3%/year	OR: 2.9 (1.9–4.5)	Age, UA, BP HbA1c, microalbuminuria	[16]
Uric acid	T1	263	N	18.1	Serum	Ma	HR: 2.9 (1.2–6.8)	HbA1c, BP, AER, serum creatinine and cholesterol	[21]
Uric acid	T1	324	N	6	Serum	Mi or ma	OR: 1.8 (1.2–2.8)	HbA1c, AER	[22]
Uric acid	T1	355	eGFR > 60 – High N and Mi	6	Serum	eGFR decline > 3.3% per year	HR: 1.4 (1.1–1.8)	Sex, ACR, HbA1c, eGFR	[23]
Uric acid	T2	1449	eGFR > 60 without Ma	5	Serum	Incident CKD	HR: 1.20 (1.03–1.57)	Age, sex, BMI, smoking, ΔHbA1c, eGFR, DM duration, ΔBP, AER, TxT	[24]
Uric acid	T1	13,964	eGFR > 60—N	4	Serum	(1) eGFR < 60	(1) RRR 2.61(1.98–3.42); (2) RRR 1.54 (1.13–2.09)	Multiple	[25]
						(2) eGFR < 60 and Ma	5th vs.1th quartile		
Uric acid	T1	3895	N, Mi, and Ma	7	Serum	(1) Albuminuria progression	(1) NS	Age, sex, BP, TG, HbA1c, DM duration eGFR	[26]
						(2) Change of CKD stage: (a) CKD 1/2 → 3/4/5; (b) 1/2/3 → 4/5; (c) 3 → 4/5	(2)		
							(a): 2.79 (1.75–4.46)		
							(b): 3.04 (1.92–4.81)		
							(c): 2.23 (1.22–4.08)		
Uric acid	T1	670	N, Mi, and Ma	5–6	Serum	(1) eGFR decline ≥ 30%	(1) HR: 3.18 (1.71–5.93)	Sex, age, BMI, HDL cholesterol, smoking, HbA1c; BP, AER eGFR, TxT	[27]
						(2) CV events	(2) HR: 2.25 (1.20–4.21)		
						(3) Mortality	(3) HR: 2.58 (1.12–5.90)		

Table 1 (continued)

Biomarker	Type of DM	*n*	Baseline Characteristics	FU (yrs)	Biofluid	Outcomes	Main Results HR (95% CI),	Adjustments	Ref
Copeptin	T2	3101	Mi and Ma	6	Plasma	2×sCr, ESRD	HR: 2.97 (1.56–6.14) Macro: HR 3.33 (1.37–9.96)	Age, sex, study treatment, AER eGFR	[29]
Copeptin	T1	736	N, Mi, Ma GENEDIAB (n = 218) GENESIS (n = 518)	10.2 5	Plasma	ESRD	HR: 1.7 (1.2–2.6)	Sex, age, DM duration, eGFR, ACR, BP, BMI, HbA1c, TxT, cohort	[30]
Copeptin	T2	756	eGFR > 60 (N, Mi, Ma)	6.5	Plasma	Change in eGFR	Std β − 0.09; p = 0.03	Age, sex, eGFR, AER, SBP, cholesterol, HbA1c, smoking, BMI, DM duration	[31]
Copeptin	T2	161	Newly diagnosed T2DM eGFR 109.1 ± 36.9	12	Plasma	CKD stage 3	1.74 (0.98–3.09)	Age, sex, smoking, BMI, SBP, HbA1c, TxT, DM duration, eGFR, CV disease	[32]
KIM-1	T1	124	Ma	10	Plasma	eGFR slope	P < 0.001 in multiple regression analysis	AER, eGFR, HbA1c	[35]
		107	Ma CKD 1–3	12.5		ESRD	P < 0.01 Cox regression for KIM-1 above and below 97 pg/ml		
KIM-1	T1	462	N, Mi eGFR > 60	8	Plasma	(1) eGFR decline ≥ 3.3% per year (2) CKD ≥ 3 stage	(1) OR: 1.3 (1.2–1.5) (2) HR: 1.3 (1.1–1.4)	HbA1c, TxT, SBP, AER, eGFR, TNFR1	[36]
KIM-1	T2	1032	N, Mi	7.4	Plasma	eGFR decline > 30% in 5 yrs	TNFR1: 1.42 (1.1–1.8)	HbA1c, ACR, BMI, SBP, eGFR, ACR, and other biomarkers	[39]
TNFR-1			eGFR > 60		Urine		KIM-1: 1.41 (1.1–1.7)		
EGF/MCP-1							uEGF/MCP-1: 0.6 (0.5; 0.7)		
EGF/MCP-1	T2	83	Mi/Ma or eGFR < 60	2	Urine	eGFR decline ≥ 25% per year	HR: 0.97 (0.94–0.99)	Age, sex, BP, GFR, ACR, TxT	[38]
EGF/MCP-1	T1/T2	208	Mi/Ma or eGFR < 60	1.4	Urine	ESRD or eGFR decline > 30%	uEGF/MCP-1: 0.8 (0.7–0.9)	Age, gender, BP, eGFR, ACR	[37]

*modelled as a -0.5 fractional polynomial. N: normoalbuminuria; Mi: Microalbuminuria; Ma: macroalbuminuria; sCr: serum creatinine, BP: blood pressure; ACR: albumin/creatinine ratio; AER: albumin excretion rate; BMI: body mass index; HbA1c: glycated hemoglobin; eGFR: estimated glomerular filtration rate; m-GFR measured glomerular filtration rate; UA: uric acid; TxT: treatment; TG: total triglycerides; OD: odds ratio; HD: hazard ratio

in both T1DM and T2DM patients [29,30]. In patients with T2DM and normal renal function, copeptin also predicted an early eGFR decline leading to CKD-3 [31,32]. However, copeptin is also a biomarker of CVD [33,34] and the association with CKD-3 incidence was no longer significant after adjustment for a prior history of CVD [32].

Markers of tubular injury

Markers of tubular injury, such as Kidney Injury Molecule-1 (KIM-1), Neutrophil Gelatinase-Associated Lipocalin (NGAL), Liver-type Fatty Acid Binding Protein (L-FABP), Monocyte Chemoattractant Protein-1 (MCP-1), and Epidermal Growth Factor (EGF), can also be measured in plasma/urine. Their potential as prognostic biomarkers in DKD has been extensively investigated. However, data are conflicting and only data on plasma KIM-1 and urinary EGF/MCP-1 are encouraging.

Plasma KIM-1

KIM-1 is a type l transmembrane glycoprotein expressed on the apical membrane of renal proximal tubule cells and plasma KIM-1 levels are increased in patients with tubular injury. In a study performed on 124 T1DM patients with albuminuria, plasma KIM-1 levels above 97 pg/ml were a positive predictor of the risk of ESRD, independently of HbA1c, AER, eGFR [35]. Moreover, in 462 T1DM patients with normal eGFR and without macroalbuminuria, plasma KIM-1 predicted both early eGFR decline and progression to CKD-3, independently of systolic blood pressure (BP), HbA1c, AER, eGFR, and TNFR1 [36]. On the contrary data on urinary KIM-1 in DKD were disappointing, likely because urinary KIM-1 best reflects acute kidney injury.

Urinary EGF/MCP-1 ratio

EGF is a peptide growth factor with a protective role in kidney injury, while MCP-1 is a chemokine that promotes the recruitment of monocytes/macrophages in the kidney. A longitudinal study on 208 patients with advanced DKD showed that urinary EGF/MCP-1 ratio was associated with a reduced risk of developing the renal outcome (ESRD or 30% eGFR reduction) and performed better than EGF and MCP-1 assessed separately [37]. In 83 T2DM patients with either micro/macroalbuminuria or eGFR <60 ml/min/1.73m^2, urinary EGF/MCP-1 was a negative predictor of a rapid GFR decline, independently of blood pressure, eGFR, and albuminuria [38]. Moreover, a recent study showed that EGF/MCP-1 was independently associated with an early eGFR decline in 1,032 T1DM patients with normo/microalbuminuria [39].

Taken together these data indicates that selected candidate biomarkers can predict onset/progression of DKD. However, the translation of promising biomarkers into clinical practise requires demonstration of clinical utility and novel biomarkers must outperform currently available biomarkers. Unfortunately, novel candidate biomarkers of DKD modestly improve the accuracy of prediction compared with models that include clinical variables, eGFR, and albuminuria. Furthermore, only few studies have tested candidate biomarkers together to assess the gain in prediction achieved with each additional biomarker. A recent study showed that several biomarkers were associated with early eGFR decline in T1DM patients with normo/microalbuminuria; however, when they were analysed together, only few of them remained significant [39].

Biomarker panels have been recently proposed to ameliorate risk prediction [40,41]. Unfortunately, there is high correlation between available biomarkers and this limits the gain in prediction of biomarker panels [40,41]. Undoubtedly, multi-marker panels perform better than single biomarker, but their absolute prognostic value is still insufficient for clinical application.

The "Omics" approach

The use of high-throughput omic approaches to analyse biological samples, such as transcriptomics, proteomics, metabolomics, has the potential to significantly contribute to biomarker discovery in DKD. Omic technological platforms allow quantification of all RNAs, proteins, and metabolites present in biological samples and produce large sets of unbiased data. Data analysis generates molecular profiling that can be used for diagnosis, outcome prediction, and response to treatments. Recently, several studies have used this new approach to biomarker discovery.

Transcriptomics

Transcriptomic studies in DKD focussed predominantly on miRNAs, small non-coding RNAs that regulate gene expression via suppression of target mRNAs. MiRNAs are present in body fluids including plasma, serum, and urine. MiRNA profiling can be performed using either traditional microarray/RT-PCR platforms or RNA sequencing (RNAseq). The main difference is that the formers profile predefined sets of miRNAs, while the latter allows for full sequencing of the whole miRNoma.

The most convincing evidence that miRNAs are potential biomarkers of DKD was provided by Pezzolesi *et al*. In T1DM patients with proteinuria and normal renal function, baseline levels of four miRNAs (let-7c-5p, miR-29a-3p, let-7b-5p, miR-21-5p) predicted the development of ESRD

at follow-up, independently of HbA1c and other confounders [42]. Another small prospective study by Argyropoulos *et al*. assessed the expression of 723 urinary miRNAs in T1DM patients with normoalbuminuria. They found that 18 miRNAs were associated with the development of microalbuminuria and nine of them were used to define a miRNA signature for microalbuminuria [43]. Several other small case-control studies reported that plasma/serum miRNA profiles differed in patients with and without albuminuria [44,45]. However, given the cross-sectional design, it is unknown if these profiles can help in predicting progression.

In body fluids, miRNAs can also travel packaged within extracellular vesicles (EVs) that protect them from endogenous RNases and EV-miRNAs are particularly suitable as biomarkers as they are very stable in biofluids. Recent advances in the techniques for EV isolation make EV-miRNA analysis less difficult and more suitable for clinical application.

We were the first to assess the urinary EV-miRNA profile in T1DM patients with and without microalbuminuria. Using a Taqman miRNA array technology, we found that 22 urinary EV- miRNAs out of 377 were differentially expressed in normoalbuminuric compared with microalbuminuric patients. Validation by qRT-PCR showed that urinary EVs from individuals with microalbuminuria were enriched in miR-130a and miR-145, while their content in miR-155 and miR-424 was diminished [46]. Afterwards, several other groups reported changes in both blood and urine EV-miRNA profiles associated with albuminuria [47,48].

Recently, few studies applied the RNA-Seq technology to evaluate EV-miRNAs. Kim *et al*. found that serum EV-miRNA profile differs in T2DM patients with normoalbuminuria and micro/macroalbuminuria and miR-4449 was highly upregulated in albuminuric patients [49]. Ghai *et al*. investigated changes in miRNA profiles in urine, urinary EVs, and EV-depleted urine fractions from T1DM patients. Urinary EV-miRNAs appeared more suitable for miRNA biomarker discovery than other fractions. Moreover, urinary EV-miRNAs differed in normo- vs. macroalbuminuric patients and changes in miR-144-3p, miR-26a-5p, and miR-30c-5p were confirmed by RT-PCR [50].

Overall these data are promising; however, we need large prospective studies assessing whether promising miRNA/EV-miRNA profiles can predict hard end-points of DKD.

Proteomics

Proteomics analyse the full set of proteins present in biological fluids. Urine is the preferred specimen for proteomic biomarker discovery in renal diseases because urine is available in relatively abundant volume, urine collection is simple and non-invasive, and urines are enriched in kidney-derived proteins. The study of proteomics in DKD is in a very early stage. Available studies were performed on small numbers of patients because of the high cost of proteomics. Moreover, most studies were cross-sectional and did not adjust results for baseline eGFR, which is a major confounder, as it is strongly associated with a large proportion of the proteome. At present, the most robust and promising proteomic biomarker in DKD is the CKD-273 classifier.

In 2010, Good *et al*. identified by using capillary electrophoresis coupled to mass spectrometry (MS) 273 urinary peptides that significantly differed between patients with CKD and healthy controls. These peptides that were predominantly collagen fragments were combined into one classifier termed CKD-273 [51]. The first external validation of CKD-273 using 144 samples showed a sensitivity of 85% and specificity of 100% for the diagnosis of CKD, and these results were then confirmed in other cohorts.

Early studies in T2DM patients suggested that CKD-273 could predict both development and progression of albuminuria [52,53]. Consistent with this, a post hoc analysis on urinary samples from 737 normoalbuminuric T2DM patients from the DIRECT-2 study showed that CKD-273 predicted incident microalbuminuria over a 4.1 follow-up period, independently of other risk factors, including baseline albuminuria and eGFR [54].

In a large cross-sectional cohort of 1,190 patients (52.9% DM) with mild-to-advance CKD, CKD-273 correlated with eGFR better than albuminuria. Moreover, a prospective analysis performed on 522 individuals with available eGFR at follow-up showed that the addition of CKD-273 to albuminuria and eGFR significantly improved prediction of fast (>5 ml/min/year) eGFR decline [55]. Consistent with this, in a large cohort of 2,673 patients (77% TDM1/TDM2) CKD-273 outperformed albuminuria in predicting an early eGFR loss (>5 ml/min/year) over a 3.3 follow-up period in individual with eGFR >70 ml/min/1.73m^2, while albuminuria was superior in patients with eGFR <50 ml/min/1.73m^2 [56]. More recently, a prospective study on 1,014 normoalbuminric T1DM and T2DM patients with baseline eGFR >70 ml/min/1.73m^2 showed that CKD-273 was the best predictor of incident CKD-3 [57], independently of age, blood pressure, and eGFR.

Taken together these data suggest that CKD-273 can be suitable to identify patients at risk of developing microalbuminuria and CKD-3. The CKD273 is now commercially available and, though costs are higher than those for urine albumin testing, an economic analysis calculated that the annual use of CKD-273 for early assessment and intervention in T2DM patients can be cost-effective when used in population with high risk of complications as those related to CVD.

The CKD273 has also been proposed for the identification of subgroups of patients responsive to treatment in RCT. However, in the DIRECT-2 study, which failed to show a

benefit of candesartan in the prevention of DKD, treatment was also ineffective in the small subgroup with a high CKD-273 score [54]. The CKD-273 classifier identified subjects at risk of developing microalbuminuria in the PRIORITY trial; however, treatment with spironolactone failed to prevent microalbuminuria in the subgroup at high-risk [58]. On the contrary, in an exploratory analysis of the MARLINA-T2D trial, patient stratification using CKD-273 unmasked a trend towards reduction in renal function loss in high-risk patients treated with linagliptin [59].

Both plasma and serum are alternative biological sources for proteomic studies. However, profiling of circulating proteins is difficult to perform due to many high-abundance proteins that can mask the low-abundant ones. However, Niewcszas *et al*. recently identified in three independent cohorts of patients with diabetes an extremely robust Kidney Risk Inflammatory Signature (KRIS), consisting of 17 novel proteins enriched for TNF Receptor Superfamily members, that was associated with the 10-year risk of ESRD [60].

Metabolomics

Metabolomics assess the in vivo metabolic status through the analysis of metabolites that are small end products of biochemical processes. Metabolomic studies, which can be targeted (pre-defined metabolites) or untargeted (all metabolites), are performed using either nuclear magnetic resonance (NMR) or MS-based platforms. The former requires larger volumes of sample, but minimal sample preparation, the latter has higher sensitivity and needs smaller sample volume, but is less robust and requires sample preparation. Although metabolomics better mirror the patient molecular phenotype compared to other omics, results are difficult to interpret because of a vast array of confounders, including lifestyle, medications, and both hormonal and nutritional state [61].

Recent prospective studies explored whether global metabolic profiles could predict renal function outcomes. In T1DM, a global metabolomics profiling performed in 158 patients with proteinuria and CKD-3 [62] identified 7 metabolites (C-glycosyltryptophan, pseudouridine, O-sulfotyrosine, N-acetylthreonine, N-acetylserine, N6-carbamoylthreonyladenosine, N6-acetyllysine) that were independently associated with eGFR slope and time to ESRD. Moreover, two large studies showed that ribonic acid, branched chain amino acids, sphingomyelin and phosphatidylcholine species were associated with the renal composite outcome (eGFR decline ≥30%, ESRD, and all-cause mortality) [63,64]. In T2DM patients, Solini *et al*. recently performed screening metabolomics in serum and urine samples from 286 Italian patients. The combination (MetIndex) of three serum metabolites (C-glycosyl tryptophan, pseudouridine, and N-acetylthreonine) predicted eGFR decline and AER rise at follow-up and improved the predictivity of clinical parameters [65]. Furthermore, in 92 American Indians with eGFR>90 ml/min/1.73m^2 Afshinnia F *et al*. found that a panel of lipids (unsaturated free fatty acids and phosphatidylethanolamines, short-low-double-bond triacylglycerols, and long chain acylcarnitines) could predict a 40% decline in GFR during follow-up, providing evidence of a relationship between lipid markers of impaired mitochondrial β-oxidation and enhanced lipogenesis with DKD progression [66]. Bioinformatic tools that integrate metabolomics and proteomics data, such as MetBridge, can help identify pathways responsible for metabolite dysregulation and also provide novel target for treatment.

Biomarkers of renal function

DKD both definition and staging are based on GFR. However, GFR measurement (m-GFR), using exogenous iothalamate, iohexol or inulin clearance, is burdensome. Therefore, GFR is usually estimated (eGFR) using creatinine and/or cystatin C-based formulae, such as the MDRD and CKD-EPI equations. Moreover, serial measurements of creatinine or, even better, cystatin C over time can be used to calculate the eGFR slope and to predict DKD progression [67].

However, creatinine levels are affected by daily protein intake and muscle mass. In addition, tubular secretion of creatinine progressively increases during the course of DKD, limiting the rise in serum creatinine level and masking GFR reduction. On the other hand, cystatin C levels are elevated in patients with obesity and obesity-related conditions, including T2DM, independently of renal function. Therefore, both creatinine and cystatin C have limitations as biomarkers of renal function. A comparison of eGFR with m-GFR in over 3,500 T2DM showed that eGFR often differed from m-GFR by ±30% or more, particularly in patients with normal renal function. In addition, eGFR values incorrectly staged CKD in 30–60% of patients [68]. Recently, β-trace protein and β2 microglobulin were proposed as alternative to creatine/cystatin C to assess GFR. However, formulae based on these novel biomarkers showed no improvements in precision or accuracy versus creatinine/cystatin C-based formulae [69].

Besides identifying novel markers to estimate GFR, it would be important to develop tools to assess nephron number, mean SN-GFR, and renal functional reserve (RFR). Both SGLT2 and RAS inhibitors preserve renal function in DKD patients at least in part by reducing glomerular capillary pressure and SN-GFR. This reduction in SN-GFR causes a drop in eGFR soon after initiation of treatment. The entity of this initial fall in eGFR may provide indirect information on SN-GFR/RFR and serve as a potential marker of the subsequent rate of decline in GFR. Consistent with this, in the RENAAL trial the acute fall in eGFR in losartan-treated T2DM patients with DKD was inversely correlated

with the long-term eGFR slope [70]. RFR can also be indirectly assessed using stress tests that acutely induce hyperfiltration (high-protein meals, infusion of amino acids or dopamine). These tests assume that pre-existing consumption of the RFR will prevent a further rise in GFR during the test; however, poor standardization and high variability make difficult to interpret the results of these functional tests.

Imaging biomarkers

In the last two decades, new functional MRI techniques (fMRI) have been developed that can generate quantitative imaging biomarkers sensitive to changes in renal blood flow, tissue perfusion, oxygenation and structure (including inflammation and fibrosis). Importantly, fMRI techniques can be performed without intravenous contrast media and are thus not contraindicated in patients with reduced renal function.

Arterial spin labelling (ASL) MRI technique uses magnetic labelling of water in arterial blood as an endogenous tracer to generate maps of renal perfusion. In a small cross-sectional study, cortical renal perfusion was reduced in T2DM patients, correlated with eGFR, and markedly decreased with progression through DKD stages [71]. Diffusion weighted/tensor imaging (DWI/DTI) MRI techniques assess the degree/directionality of water movements in tissues, expressed as ADC (apparent diffusion coefficient) and fractional anisotropy (FA). As both deposition of extracellular matrix and tubular atrophy restrict the mobility of water molecules, ACD and FA have been proposed as biomarkers of renal fibrosis. A small study in T2DM patients showed that medullary FA values were significantly reduced in patients with microalbuminuria [72]. In Blood Oxygen Level Dependent (BOLD) MRI, high values of R2* (relaxation rate) indicate higher deoxyhaemoglobin concentrations and thus reduced renal tissue oxygenation. However, in patients with diabetes, R2 did not correlate with eGFR [73] and failed to distinguish patients with different stages of CKD [74]. A large prospective multi-centre observational cohort study (iBEAt-DKD) is currently enrolling patients with T2DM and eGFR $\geq$30 ml/min/1.73m^2 to clarify if renal imaging biomarkers have potential as prognostic biomarkers in DKD.

Conclusive remarks

Several traditional candidate biomarkers are independently associated with renal outcomes in DKD. However, they modestly improve the performance of currently available clinical biomarkers. Moreover, biomarker thresholds warranting clinical action have not yet been defined and the ability of novel biomarkers to improve clinical outcomes by guiding decisions/interventions has not been tested in RCT. Therefore, for the time being assessment of eGFR and albuminuria remain the cornerstone of diagnosis/risk stratification in daily clinical practice.

Novel omic approaches and integration of multiple omics data (multi-omics) have enormous potential for biomarker discovery in DKD. However, large study cohorts with kidney biopsies and both urine and plasma/serum samples from the same patients are needed to adequately perform integrative multi-omics studies. Consensus protocols for sample collection, processing, and analysis should be defined to obtain comparable and reproducible data across studies. Finally, both analysis and interpretation of results will require specialized bioinformatic tools to turn big data collection into biomarker discovery.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors

Informed consent For this type of study, formal consent is not required.

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