

Urinary Complement proteome strongly linked to diabetic kidney disease progression

Corresponding Author: Dr Monika Niewczas

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The present study examined the urinary proteome in four prospective cohorts of 833 subjects with either type 1 or type 2 diabetes and found that the Complement system represented the most enriched pathway. Independent associations between complement and kidney failure development in 10 years were very strong. Kidney tissue transcriptomic data showed a strong concordance with urinary findings. Altogether, these data support the involvement of Complement system in diabetic kidney disease progression.

This is a nicely done study. Although the concept that complement is implicated in DKD progression is not novel, the data provided here significantly add to the field. There are issues, however, that should be carefully concerned:

1. The tissue analyses focused on complement gene expression. These data point at a local production of complement components, but histological staining for complement split products (in a subset of patients) would help understanding the renal cells that are most targeted by complement effector mechanisms.
2. The protein expression of local complement regulators (e.g., DAF and CD59) by different cells would also help deciphering the role of complement in disease pathophysiology.
3. The reviewer would appreciate having a clearer understanding of how the complement is activated in DKD and what are the effector mechanisms. Is the correlation with albuminuria due to the fact that complement is freely filtered in albuminuric patients or is complement damaging podocytes, which leads to albuminuria?

Reviewer #2

(Remarks to the Author)

REVIEW SUMMARY

What are the noteworthy results? Yes, the authors performed high-throughput and deep profiling of urine of individuals with diabetes (both types) to identify putative biomarkers that are associated with future risks of kidney failure or worsening function.

Will the work be of significance to the field and related fields? Yes, the authors have extended their previous work on plasma profiling using somascan technology to highlight an inflammatory signal in urine. This may offer a more focal point of injury in the kidney and urinary system.

How does it compare to the established literature? If the work is not original, please provide relevant references.

Does the work support the conclusions and claims, or is additional evidence needed?

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

Is the methodology sound? Does the work meet the expected standards in your field? For the most part, yes. The authors can do more with regards to clarity, as details on the study design (e.g., cohort study, patient selection, inclusion/exclusion criteria, etc) were not easy to find.

Is there enough detail provided in the methods for the work to be reproduced? Aptamer-based proteomics is an exciting new field. SOMAscan boasts an extensive coverage of the proteome, however their menu of proteins is not so easily accessible. Briefly, how many proteins are included in the 1.1k kit, how many were quantified and at what rate (e.g., missingness rate)?

COMMENTS

In the current study, the authors examined the urinary protein profiles of 833 individuals with DKD and assess associations

between key proteins and kidney failure/decline. They identified proteins from the complement pathway to carry the strongest associations for kidney failure development, independent of clinical factors. They also link urinary profiles with those from plasma and kidney tissue; showing stronger concordance between the urine and kidney (compared to plasma and kidney). The study offers an importance extension of the authors' previous work on the circulating profiles (in which they identified inflammatory players connected to kidney outcomes) and also show that the urinary profile may be more reflective of the kidney milieu than its circulatory counterpart. Overall, this study provides exciting insights for further study into the roles of the inflammatory pathways in progressive diabetic kidney disease.

There are however some weaknesses in the present form of the work that must be addressed before publication.

MANUSCRIPT

First, I would appreciate more details on the study design in the introduction or results. How many total patients are enrolled in the Joslin Kidney Study? Were they all included in the current study? If not, what were the inclusion/exclusion criteria? Like with the 207 T1D subjects from the PERL trial (which has 530 participants)? It is also unclear whether the authors measured the top% proteins in all patients across urine, plasma, and kidney tissues.

It does not seem appropriate to write that there are four cohorts, the majority (189+115+322) of patients are from the Joslin Kidney Study. This may mislead readers into thinking the study population is more diverse than it is. In table 1, it may be more informative for readers to clinical characteristics of the four "cohorts" separately since the incidence of kidney failure is different between those with T1D (53%) vs T2D (23%) in the advanced disease group. (This aligns with how the authors presented Figure 4 and may be a more suitable way to show the differences/similarities between types of diabetes and among stages of DKD.) How the profiles differ between T1D vs T2D within the discovery group? Is complement pathway activation more apparent in one group over the other?

The SOMAscan assay is a rapidly advancing one. I appreciate that the authors provide extensive descriptions of the technology and would like more information on the 1.1 kit (as this is not so easily accessed online). How many proteins are included in the kit? Since SOMAscan was originally developed for plasma/serum, how many proteins from the kit could be quantified in urine (do the authors perform any filtering to remove proteins with missing values)?

How do the albuminuria categories compare within the advanced DKD group, between incident kidney failure cases and non-incident controls? In Figure 5a – the differences between those who do and do not advance to kidney failure are quite apparent at baseline in the urinary and kidney tissue measurements, but not in circulatory measurements. Since these proteins are quite large, it is possible that the increased excretion of these complement proteins may arise from a more compromised filtration barrier in those who eventually developed kidney failure. Figure 3b would demonstrate that these urinary proteins are strongly correlated with ACR. Therefore, the authors should be careful in discussing the engagement of complement in the kidney, as these proteins may merely reflect ACR and systemic inflammation (rather than renal inflammation).

The overall discussion is too long. At times, it goes off-topic as it dives too deep into the therapeutic implications rather than on the findings themselves. The 2nd paragraph of the discussion discusses the therapeutic landscape. Certainly, these complement proteins may be novel targets. Given that the study included PERL trial subjects, how does allopurinol influence the protein profiles (in urine, circulation, and kidney)? For all patients, how do other medications (and their duration) influence these associations? Are any of the 833 subjects on anti-inflammatory medications?

A major limitation of the work is the lack of diversity. The participants in the Joslin studies are notoriously White (80%+). The PERL trial is also predominantly White. Historically, the authors have included the Pima Indian population; these individuals may serve as an interesting validation/extension cohort. Whether these complement proteins are also key predictors of kidney failure in non-White populations is worthy of discussion and further study. Including this would significantly enhance the current work; at the very least, it should be discussed as a limitation.

FIGURES

Overall, given the four "cohorts", it can be confusing to the reader to figure out which "cohorts" were examined in each figure. The authors can provide more details within the figure for improved clarity.

Figure 2a: More informative to add protein names on x-axis, as it was not easily understood that each needle represented a protein with significant risk.

Figure 2b: to be consistent, authors should keep the same axes for focused needle plots (ie., x = proteins, y = pvalue) as the broader one in Fig 2a. Alternatively, consider moving 2A into the supplementary. Are any of these proteins from figure 2A downregulated?

Figure 3: There seems to be a typo in the caption as it refers to "Figure 2a, c." More explanation is required. This figure was confusing and hard to understand. What is included in M1?

Figure 4a: shows good agreement of protein risk between the 2 types of diabetes, in terms of significance. Why did the authors correlate p values and not the actual risk variable (are the magnitudes similar? Direction of risk the same?)

It may flow better with the work (discovery to extension) in presenting Figure 5 before Figure 4.

WRITING

Certain words such as "complement," "extension," "discovery."

The authors do not write out "DKD" in the first paragraph of the introduction.

Discussion is quite long, with the first sentence being too wordy.

Consider reworking the title, as “four cohorts” may be misleading.

Reviewer #3

(Remarks to the Author)

Zaipul I. Md Dom et al. carried out biostatistical analyses of urinary proteome in four prospective cohorts of 833 subjects with either type 1 or type 2 diabetes in an attempt to understand the progression of diabetic kidney disease and to obtain potential biomarkers. They obtained interesting findings that the most enriched pathways belonging to the Complement system. One strength of the study is that they use four 10-years follow-up cohorts of subjects with type 1 and type 2 diabetes. The other strength of the study is that they compared the results across three specimens, namely urine, plasma, and kidney tissues, to reveal the source of the biomarkers (Complement proteins). However, there are some concerns and unsolved issues as mentioned below.

Major points:

1. Table 1. The gender of the subjects is not balanced within the same group, namely within the advanced DKD (female : male = 44% : 56%) or within the early-to-moderate DKD (female : male = 30% : 70%), as well as between the two different groups.
2. Diabetes type is a concern. In the advanced DKD (type 1 : type 2 = 62% : 38%); in the early-to-moderate DKD (type 1 : type 2 = 39% : 62%). Since type 2 diabetes is usually insulin-resistant while type 1 is insulin-dependent, they have different latency (Table 1) in developing DKD and associate with different molecular pathogenesis. In the very beginning, if the authors separate the type 1 and type 2 patients and separately carry out the analyses, the results are expected to be different. For example, they may find different Complement protein and/or different ranking that associated with type 1 and type 2. They may also find features (markers) unique to type 1 and type 2, as well as features common to both type 1 and type 2. In the present results of the manuscript, what the authors found are features (markers) common to both type 1 and type 2 since they started with a collection combined both type 1 and 2 patients. Data adjusted for clinical covariates (e.g. sex, diabetes type) is a statistical method, which is different from an analysis using separate patient groups of type 1 and type 2 in the beginning.

Minor points:

Figure 2a, the needle plot, on the x-axis, proteins are ordered by the pathway, the UniProt ID and the protein name. Some of the color between groups are too similar to tell the difference. In addition, one vertical needle represents P value of one Complement protein. The authors are suggested to indicate the name of each needle.

Reviewer #4

(Remarks to the Author)

This manuscript by Dr. Niewczas and colleagues summarizes findings from an association study linking urine protein levels to incident adverse kidney outcomes (kidney failure, a composite endpoint of kidney failure and at least 40% loss of eGFR, and decline in eGFR) in four prospective studies of participants with type I and type II diabetes. The authors chose a discovery-validation design with each one type I and one type II diabetes collective, and quantified levels of ~1,300 urine proteins with Somascan aptamer technology. Urine protein levels were compared to their corresponding plasma and kidney tissue counterparts, as well as to classical molecular markers of kidney injury and diabetic kidney disease (DKD). The authors find that proteins belonging to the complement pathway are strongly enriched in persons reaching the kidney endpoint, and that high levels of these proteins in urine predict adverse kidney outcomes better and are linked to the kidney proteome much better than their plasma counterparts. The results suggest the importance of the complement system in the progression of DKD.

The manuscript is well written, and deals with a clearly important and increasingly common public health concern, DKD. The authors are to be commended on the comprehensiveness of their study design, including multi-matrix protein quantification, validation of aptamer quantification with orthogonal technologies, comparison of type I and type II DKD, and a comparison of classical biostatistical approaches to data analysis with newer machine learning methods. However, there are also limitations.

The major limitations of this study is the lack of an independent replication sample, in which to confirm associations and assess the predictive power of the urine protein biomarkers. The discovery results from this study are not independently validated. The study design is presented in a misleading manner (e.g., presenting this as a four cohort design, when in fact only selected discovery proteins were quantified in the extension with a different technology). Comments about these points, as well as other limitations, are listed below, along with some suggestions:

Results:

1. Page 5, Figure 1: the authors state that the figure provides an overview of the study framework, but in fact the figure in its current state does not provide a very informative overview of the study design. The figure should include the sample sizes of each sample. Further, the figure needs to show which measurements and which analyses are performed in both discovery and extension, and which only in the discovery cohort. It is important that readers are informed whether and which findings were independently assessed for validation in the extension study.

2. On page 9, lines 170 ff: please clearly state if plasma and kidney tissue proteins were quantified in all participants of the discovery cohorts. This can be included as sample size information in the revised figure 1, it should not just be hidden in a supplementary table.
3. Each main figure should contain information in the figure panels (e.g., as part of the title of axis labeling) whether it is based on the discovery study only. It is very misleading if the authors present this as a four cohort validation – extension study design, when in fact only secondary analyses included information from the extension study, and only for six out of the 1300 proteins.
4. Still along the same lines, only Figure 4 b and c seems to contain data from the extension cohort. Only at the very end of the manuscript (page 30, Methods), did I find information that in fact the extension cohort only performed targeted measurements of complement proteins and not across the urine proteome. This does not become clear in the text. Figure 1, abstract and manuscript need to be rephrased as a two-study discovery cohort only design. The two-study extension is only a secondary analysis and should therefore not be shown on the same level as the discovery.
5. The plots in Fig 4b and c show that the associations with annual eGFR slope and with the composite kidney outcome are clearly attenuated in comparison to the discovery cohort. I could not find easily if these results are from the base or the adjusted model. They should show results from the adjusted model. Please clarify and include adjusted results, if not adjusted already.
6. The authors interpret the attenuated associations in the extension in Figures 4b and 4c to mean that associations in advance kidney disease are stronger than in early-to-moderate DKD, but in fact it may just be winner's curse or related to the different assays that was used in discovery and extension. In support of winner's curse, it is quite unusual that the prediction of outcomes at 10 years (i.e, more distantly in the future) performs better than at 3 or 5 years, as shown on page 10, 198 ff. How do the authors explain this?
7. Page 6, lines 118 ff: please provide the adjusted results that correspond to the unadjusted results in lines 101 ff.
8. In order to interpret the enrichment in complement markers in the discovery better, it is important to know their correlation. Please include a correlation matrix between the 66 significant proteins from the discovery cohort in a supplementary table and visualize as a correlation matrix sorted by pathway. Please provide such plots stratified by albuminuria category (A2, A3) at baseline.
9. Page 9, lines 185 ff: in this Reviewer's opinion, the single cell data don't add important information to the manuscript and can be removed. The authors state themselves in the introduction that the transcriptome and the proteome are poorly correlated.
10. Page 10, lines 203 ff: the results of the clustering analyses are driven by the fact that they are based on proteins that are associated with outcomes in the same sample. The prognostic power (composite endpoint) should be assessed in the extension study, for the markers available there.
11. Discussion: the discussion of the findings is long, but the lack of independent replication, a major limitation, is not adequately addressed. Many of the details can be shortened, as the findings from this first urine proteomics study need to be validated in an independent replication sample first.

Minor comments:

1. In Table 1, please include information about race.
2. Page 6, line 107: it seems unlikely that only 14 persons died over the course of follow-up (10 years). Please double check.
3. Page 11: the results end quite abruptly, consider including one summarizing sentence.
4. Figure 4 a: putting the base and adjusted estimates side by side would be better to make apparent if their confidence intervals overlap.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors adequately addressed my concerns.

Reviewer #2

(Remarks to the Author)

I am pleased with the revisions. The authors have put in tremendous work to fulfill the diversity aspect by including and collaborating with the Diabetes Heart Study of African American study.

Reviewer #3

(Remarks to the Author)

The authors have revised the content of the manuscript based on the points raised by this reviewer. The reviewer supports the publication of this revised manuscript without further question.

Reviewer #4

(Remarks to the Author)

The authors have thoroughly revised the manuscript, and added an external validation sample, as requested. I note that the

effect estimates with respect to slope and composite kidney endpoint in this new external sample, while concordant in direction, are smaller than in the other two early DKD studies for the tested top proteins and also smaller than in advanced DKD. Several of them are not even statistically significantly associated with adverse kidney events in this new sample (see Fig. 4b). Moreover, the three early DKD validation cohorts do not adjust their Cox models for albuminuria. Hazard ratios in 4a still seem to be presented unadjusted, which may be misleading. I suggest updating 4a to show adjusted estimates to be concordant with the other panels, and to adjust the early DKD cohorts for UACR as well, even if not all cohorts are feasible. This reviewer believes it is important to give a realistic depiction of how much these urinary proteins can add above and beyond UACR. Other than this point, the authors have adequately responded to my comments, and their efforts are very much appreciated.

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RESPONSES TO THE REVIEWERS

We would like to thank all the Reviewers for the comprehensive review of our manuscript. We believe the revision resulted in much improved content and that will ultimately benefit the reader.

Following is the list of the major changes made to the revised manuscript.

- **Independent replication cohort.** In response to critiques of the Reviewers #2 and #4, we acquired clinical data and samples from the Diabetes Heart Study of African American subjects with T2D and early DKD. We performed measurements of top Complement proteins and we very gladly observed that the effects are replicated. The project was performed in collaboration with **Drs. Freedman and Palmer**, Wake Forest, now co-authors of our manuscript.
- **Refinement of the study design.** In response to critiques of Reviewers #2, #3 and #4, we refined the presentation of our study design that resulted in multiple changes; new Fig.1 on study design, updated nomenclature describing cohorts (advanced and early-to-moderate DKD cohorts, no longer described as discovery and extension), an **updated title**: “*Urinary Complement proteome is strongly linked to diabetic kidney disease progression*”, an updated Suppl. Table 11 on Study representativeness and numerous changes throughout the manuscript text and in displays.
- **Multiple new analyses.** In responses to a number of critiques, we now provide detectability data for proteomics, adjusted models for intermediate kidney outcomes, expanded targeted measurements in advanced DKD, correlations with the molecular weight, clustering in early DKD stages, analyses of mortality data, kidney tissue histology indices among others.
- **Discussion.** To address multiple valid comments of the Reviewers, the discussion has been substantially reshaped.
- **Spatial transcriptomics.** In order to inform the study further in terms of tissue localization of proteins of interest in response to critiques of the Reviewer #1, we are adding data generated with a cutting edge technology of spatial transcriptomics for the genes corresponding to top proteins. We gladly saw, the expression is markedly increased in DKD cases.

The major changes in the text are outlined in yellow, whereas major changes in displays are marked in red.

Reviewer #1 (Remarks to the Author):

The present study examined the urinary proteome in four prospective cohorts of 833 subjects with either type 1 or type 2 diabetes and found that the Complement system represented the most enriched pathway.

Independent associations between complement and kidney failure development in 10 years were very strong. Kidney tissue transcriptomic data showed a strong concordance with urinary findings. Altogether, these data support the involvement of Complement system in diabetic kidney disease progression.

This is a nicely done study. Although the concept that complement is implicated in DKD progression is not novel, the data provided here significantly add to the field.

Thank you for your kind comments on our study, acknowledging that our data significantly add to the field.

R1Q1. There are issues, however, that should be carefully concerned: The tissue analyses focused on complement gene expression.

R1Q2. These data point at a local production of complement components, but histological staining for complement split products (in a subset of patients) would help understanding the renal cells that are most targeted by complement effector mechanisms.

That is correct. Our kidney tissue analyses offer data for the complement **gene expression data** corresponding to the top 5% proteins. As the reviewer rightfully pointed out, these data are **novel** as they point to the local production of the complement, contrary to the overall notion in the field that has been focusing on systemically produced complement potentially affecting kidney. Noteworthy, our gene expression data are based on the new **single cell transcriptomics (sc/snRNAseq) technique** allowing us to obtain high-resolution **insights into the cellular sources**.

We absolutely agree that evaluation of the split products is important. We would like to bring to the Reviewer's attention that **our tissue analyses** included the **aptamer proteomics (Suppl. Table 4)**. This technology that we chose for our study, has unique capabilities to **measure intact and split products** (C3 and C3a or C5 and C5a or opsonins, respectively). It is, indeed, interesting that we observed a strong signal for C5a in the urine, whereas the differences by caseness at the protein level were more prominent for C5 (intact) than for C5a in the kidney tissue. In that sense, the aptamer technology is superior to the mass spectrometry, which, by the method principle, cannot distinguish intact from split products. Please note that ours is the **first-in-class** such a **comprehensive evaluation of the kidney tissue Complement at the protein level**. It is true that aptamer proteomics does not allow us to speak to the exact tissue location. We have explored such a possibility, but laboratory protocols for microdissection of glomerular and tubular compartments are not compatible with downstream aptamer proteomics.

To further investigate the tissue localization of the involved Complement, we provide the following **new results**:

- Associations of the **Complement protein expressions** generated with aptamer proteomics **with (new) detailed histology indices** of the kidney injury: (interstitial fibrosis, lymphocytic infiltrate, plasmacytic infiltrate, eosinophils, intimal fibrosis, arteriolar hyalinosis, globally sclerotic glomeruli). Please see the **updated Table S8** with the histology by caseness and a **new Suppl. Table 5** focusing on the relationships with our top Complement proteins and split products. Interestingly, C7 protein expression in the kidney tissue correlated strongly with interstitial fibrosis and immune infiltrates, whereas C5, C3 or derived anaphylatoxin correlated markedly with the intimal fibrosis.
- To appreciate the tissue localization, we take advantage of powerful technology - **spatial transcriptomics** and we present **new data** for our top Complement proteins (**new Suppl. Fig. 3**). We absolutely recognize the importance of the **immunostaining** for Complement. There are a few studies in the literature reporting an increased C5a or C3c immunostaining in diabetic kidney tissue.¹⁻³ We agree, that those types of projects should be pursued in the future on a larger scale. Some potential caveats of immunostaining may be non-specific binding by sclerotic glomeruli,⁴ a lack of certainty whether the Complement presence is due to kidney production or a result of being uptaken from circulation among others.⁵ The latter may be partially overcome by our RNA-based data. Accordingly, we have expanded the Discussion and added new references on this topic.

R1Q3. The protein expression of local complement regulators (e.g., DAF and CD59) by different cells would also help deciphering the role of complement in disease pathophysiology.

We absolutely agree that the mentioned molecules: DAF (CD55)⁶ or CD59⁷ are considered important players in the biology of diabetic complications including kidney disease.

Notably, in our study DAF in circulation was the most strongly associated Complement protein with the kidney outcome. Concordantly, in our former report (*Niewczas et al. Nat Med 2019*),⁸ DAF was among the most significant inflammatory proteins in circulation associated with the kidney failure risk (it was a member of KRIS – Kidney Risk Inflammatory Signature). Nevertheless, associations of DAF in the urine or kidney tissue were weak.

To appreciate its relevance, we now indicate DAF on our needle plots (urine, plasma) and on the volcano plot (kidney tissue); **updated Fig. 4**.

CD59 is another critical component of the Complement activity that inhibits terminal complex formation by binding to C8 and preventing C9 from joining the complex. Its importance has been implicated as a biomarker of diabetic complications including diabetic kidney disease. In our data, CD59 was not associated with the prospective kidney outcome. The differences may be potentially attributed to different proteomics technologies in ours (aptamer) and other studies (mass spectrometry or antibody based) or capabilities to detect the glycosylated form, in particular.^{7,9} Of potential interest, it could be that CD59 was particularly correlated with C8 ($r=0.65$) by contrast to correlations with other proteins of the complex ($r\leq 0.33$). See the **new Suppl. Table 10** that presents correlations among all the Complement proteins, provided also in the response to R4Q8.

For the Reviewer's reference we provide here **new sc/snRNAseq data** for DAF and CD59. It appears that DAF is enriched in infiltrating immune cells (aligning with the strong data in circulation, but not in urine), while CD59 is enriched in endothelial and tubule cells, but with varying expressions by case. We opted not to include these data into the manuscript as the two molecules were not among the top signals in our urinary proteomics, which was our primary driver (**new Fig. R1**).

R1Q4. The reviewer would appreciate having a clearer understanding of how the complement is activated in DKD and what are the effector mechanisms. Is the correlation with albuminuria due to the fact that complement is freely filtered in albuminuric patients or is complement damaging podocytes, which leads to albuminuria?

That is a very important question. In our humble opinion, the phenomenon we observed in our study is not due to the fact of Complement being freely filtered in subjects with albuminuria. The Complement is likely being produced locally in the diabetic kidney. This notion is supported by our **new Suppl. Fig. 2**, which presents the associations of the Complement proteins with the kidney outcome by molecular weight demonstrating no significant pattern between the two ($p=0.54$). Other, multiple lines of evidence supporting local production over filtration in the paper include:

- urinary Complement associations are much stronger than the circulating associations;
- differential expression (increased) of Complement proteins in the kidney tissue;
- single cell and spatial RNA-based data.

Reviewer #2 (Remarks to the Author):

REVIEW SUMMARY

What are the noteworthy results?

Yes, the authors performed high-throughput and deep profiling of urine of individuals with diabetes (both types) to identify putative biomarkers that are associated with future risks of kidney failure or worsening function.

Will the work be of significance to the field and related fields?

Yes, the authors have extended their previous work on plasma profiling using SOMAScan technology to highlight an inflammatory signal in urine. This may offer a more focal point of injury in the kidney and urinary system.

How does it compare to the established literature? If the work is not original, please provide relevant references. Does the work support the conclusions and claims, or is additional evidence needed?

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision? Is the methodology sound? Does the work meet the expected standards in your field?

For the most part, yes.

The authors can do more with regards to clarity, as details on the study design (e.g., cohort study, patient selection, inclusion/exclusion criteria, etc) were not easy to find.

Is there enough detail provided in the methods for the work to be reproduced? Aptamer-based proteomics is an exciting new field. SOMAScan boasts an extensive coverage of the proteome, however their menu of proteins is not so easily accessible. Briefly, how many proteins are included in the 1.1k kit, how many were quantified and at what rate (e.g., missingness rate)?

COMMENTS

In the current study, the authors examined the urinary protein profiles of 833 individuals with DKD and assess associations between key proteins and kidney failure/decline. They identified proteins from the complement pathway to carry the strongest associations for kidney failure development, independent of clinical factors. They also link urinary profiles with those from plasma and kidney tissue; showing stronger concordance between the urine and kidney (compared to plasma and kidney). The study offers an importance extension of the authors' previous work on the circulating profiles (in which they identified inflammatory players connected to kidney outcomes) and also show that the urinary profile may be more reflective of the kidney milieu than its circulatory counterpart. Overall, this study provides exciting insights for further study into the roles of the inflammatory pathways in progressive diabetic kidney disease. There are however some weaknesses in the present form of the work that must be addressed before publication.

Thank you very much for these acknowledgements of our study.

MANUSCRIPT

R2Q1. First, I would appreciate more details on the study design in the introduction or results. How many total patients are enrolled in the Joslin Kidney Study? Were they all included in the current study? If not, what were the inclusion/exclusion criteria? Like with the 207 T1D subjects from the PERL trial (which has 530 participants)? It is also unclear whether the authors measured the top% proteins in all patients across urine, plasma, and kidney tissues.

Thank you for these comments. Please see **new Suppl. Fig. 1a and b** that present **flow charts** of the inclusion criteria of study subjects coming from the Joslin Kidney Study or PERL and the corresponding description in the Supplementary methods.

We also now explicitly state in the **updated Fig. 1**, which method was utilized for which samples. The top 5% proteins in the advanced DKD cohorts were measured with aptamer proteomics, whereas the same proteins in the early/moderate DKD cohorts were measured with targeted methods. In response, we have expanded targeted measurements in the advanced DKD cohorts for select proteins. Associations with the kidney outcome were similar between the two methods (**new Suppl. Fig. 4**). Please see also our orthogonal comparison between the two methods in the study subset (**Ext. Data Fig. 9**).

R2Q2. It does not seem appropriate to write that there are four cohorts, the majority (189+115+322) of patients are from the Joslin Kidney Study. This may mislead readers into thinking the study population is more diverse than it is. In table 1, it may be more informative for readers to clinical characteristics of the four "cohorts" separately since the incidence of kidney failure is different between those with T1D (53%) vs T2D (23%) in the advanced disease group. (This aligns with how the authors presented Figure 4 and may be a more suitable way to show the differences/similarities between types of diabetes and among stages of DKD.)

We agree with the Reviewer. We now present clinical characteristics of each cohort separately in **the updated Table 1**.

To address an issue of diversity, we added a **new, independent cohort** of **Diabetes Heart Study** participants, African American subjects with T2D as a third cohort with early-to-moderate DKD. Complement associations in this early/moderate DKD cohort appear similar to the two other early/moderate cohorts (PERL in T1D and Joslin Kidney Study in T2D); **updated Table 1, updated Fig 4. and Suppl. Table 6**.

R2Q3. How the profiles differ between T1D vs T2D within the discovery group? Is complement pathway activation more apparent in one group over the other?

Thank you for bringing this aspect to our attention. An important factor driving our decision was our goal to preserve the power of this high-throughput proteomics study. Importantly, as we formally tested, there was no interaction between the top Complement proteins and the diabetes type in our models.

We very much agree that there may be protein associations highly specific to diabetes type, which may be missed by design in our study prioritizing DKD stage over diabetes type. Larger studies, specifically designed for comparisons between type 1 and type 2 may be conducted in future. We provide these auxiliary data on the Complement proteome by diabetes type in **updated Fig. 4 and Suppl. Table 4**.

Interestingly enough, in the nephrology field a notion exists that there are shared commonalities among subjects with chronic kidney disease of different etiologies (type1 or type 2 diabetes or other) as the disease advances. Concordantly, evidence is emerging that the molecular phenotypes of kidney disease progression are similar between type 1 and type 2 diabetes and other chronic kidney disease etiologies as reported by us and others.^{8,10,11} We expanded our comments in the **updated Suppl. Table 11** on the study's generalizability and in the Discussion on study limitations.

R2Q4. The SOMAscan assay is a rapidly advancing one. I appreciate that the authors provide extensive descriptions of the technology and would like more information on the 1.1 kit (as this is not so easily accessed online). How many proteins are included in the kit? Since SOMAscan was originally developed for plasma/serum, how many proteins from the kit could be quantified in urine (do the authors perform any filtering to remove proteins with missing values)?

Thank you for these valid comments. We added a **new Suppl. Table 12** that lists all the proteins measured.

We have now also added **new information on the detectability** of the proteins in our samples. Importantly, our top 5% proteins were all very-well-detectable. Overall, 83 out of 110 Complement proteins were very-well-detectable (those were detected in $\geq 70\%$ of our samples; in other words, they were detected in more than two tertiles of the protein distributions allowing for our analysis per tertile change to be valid). Cox results for non-very-well-detectable 27 proteins are now marked in italics and explained in the legend. We analyzed further the 27 proteins with a Fisher exact test. Well-detectable proteins, 13 out of 27 (those detected in $\geq 50\%$ of the samples from our study population) were treated as a categorical variable above/below median, whereas the remaining 14 non-well-detectable proteins were categorized based on detection; the latter results of the Fisher tests are marked in italics (**Methods and updated Suppl. Table 4**).

R2Q5. How do the albuminuria categories compare within the advanced DKD group, between incident kidney failure cases and non-incident controls? In Figure 5a – the differences between those who do and do not advance to kidney failure are quite apparent at baseline in the urinary and kidney tissue measurements, but not in circulatory measurements. Since these proteins are quite large, it is possible that the increased excretion of these complement proteins may arise from a more compromised filtration barrier in those who eventually developed kidney failure. Figure 3b would demonstrate that these urinary proteins are strongly correlated with ACR. Therefore, the authors should be careful in discussing the engagement of complement in the kidney, as these proteins may merely reflect ACR and systemic inflammation (rather than renal inflammation).

We absolutely agree that it is a very important question whether the Complement proteins in the urine associated with increased kidney risks are due to an increased kidney production, due to systemic inflammation or simply due to the increased leakage. We discuss a few lines of evidence that support the idea of the kidney production:

- in our new analyses there is no correlation between the molecular weight of the proteins and the strengths of the associations with the kidney outcome (**new Suppl. Fig. 2**).
- our kidney tissue proteomics shows that a number of Complement proteins have an increased expression in diabetic kidney tissue (**Fig.4**);
- we demonstrated that diabetic kidney is capable of producing Complement proteins and a number of them are increased in diabetic kidney (based on single cell data and on the new spatial transcriptomic data (**Ext. Data Fig 4 and new Suppl. Fig. 3**);

- RNA-based evidence from the literature referenced in the discussion.

There were 59% subjects in A3 albuminuria category and 17% subjects in A2 albuminuria category, respectively, who developed kidney failure in 10 years in the advanced DKD cohort. Median (25th, 75th percentile) of the ACR value for subjects with an incident kidney failure was: 1516 (654, 2632) mg/g creatinine, whereas it was 172 (43,662) mg/g creatinine for non-incident subjects. The ACR values in subjects with an incident kidney failure were higher, but the interquartile ranges overlap. Please see also responses to **R1Q3**.

R2Q6. The overall discussion is too long. At times, it goes off-topic as it dives too deep into the therapeutic implications rather than on the findings themselves. The 2nd paragraph of the discussion discusses the therapeutic landscape. Certainly, these complement proteins may be novel targets.

We shortened the discussion of the therapeutic implications. Please note that the reason the overall word count did not decrease is due to the other text additions in response to other critiques of the reviewers.

R2Q7. Given that the study included PERL trial subjects, how does allopurinol influence the protein profiles (in urine, circulation, and kidney)? For all patients, how do other medications (and their duration) influence these associations? Are any of the 833 subjects on anti-inflammatory medications?

There was no interaction between the top Complement proteins and allopurinol treatment in our models. This information has now been added to the Results. Please also note that the results of the original intervention in the PERL study have been negative.

Nevertheless, it is potentially a valid consideration. Uric acid has been linked to complement and inflammation in general,^{12,13} although most of the data focused on gout or other diseases. There are ongoing projects pursued by PERL study leaders and their collaborators that will shed more light on potential relationships between allopurinol treatment and circulating inflammatory biomarkers.

It may potentially have been possible to evaluate the anti-inflammatory medications in the most recent study population, which is PERL. Regretfully, due to the historical nature of the remaining cohorts, it is not possible for us to reconcile the medications and offer a fair evaluation of this subject.

We comment on that in the **updated Suppl. Table 11**.

R2Q8. A major limitation of the work is the lack of diversity. The participants in the Joslin studies are notoriously White (80%+). The PERL trial is also predominantly White. Historically, the authors have included the Pima Indian population; these individuals may serve as an interesting validation/extension cohort. Whether these complement proteins are also key predictors of kidney failure in non-White populations is worthy of discussion and further study. Including this would significantly enhance the current work; at the very least, it should be discussed as a limitation.

We very much agree. We have added to our study another **independent cohort** comprising **African American** subjects, participants of the **Diabetes Heart Study** (DHS) with T2D. The DHS had made highly relevant important contributions to the field regarding the course of the diabetic complications in this population.¹⁴⁻¹⁶ We were excited to see that the results seemed replicating the other two early/moderate DKD cohorts. Please refer to the **updated: Table 1, Fig. 4 and Suppl. Table 6**.

Pima Indian or American Indian population has been an observational study that fundamentally contributed to our understanding of the diabetic kidney disease progression from epidemiological and molecular perspective.¹⁷ It also included circulating biomarker studies performed together with the Joslin Kidney Study cohorts.^{8,18} Regretfully, the urine specimens from the Pima Indian population are not available for this study. We expanded comments on diversity in the **updated Suppl. Table 11 and in the Discussion**.

R2Q9. Figures. Overall, given the four “cohorts”, it can be confusing to the reader to figure out which “cohorts” were examined in each figure. The authors can provide more details within the figure for improved clarity.

We have carefully reviewed all figures and have made improvements to enhance their clarity following your valuable feedback. To address your concern about identifying the cohorts examined in each figure, we have revised the legends accompanying each figure to explicitly state which cohorts are represented. In addition, we have added clear annotations directly on each figure to indicate the corresponding cohorts. These annotations should provide immediate context to the reader.

Figure 2a: More informative to add protein names on x-axis, as it was not easily understood that each needle represented a protein with significant risk.

Thank you for this comment. We have thoroughly considered your suggestion to add protein names on the x-axis of Fig. 2a. However, we were not successful in identifying an approach that would effectively present the names of all 110 proteins while preserving sufficient clarity. Alternatively, we have prepared a **new Suppl. Table 10**, also in response to **R4Q8**, which lists all 110 Complement proteins in the same order as presented in Fig. 2a. We are referring to this table in the Fig. 2 legend.

Figure 2b: to be consistent, authors should keep the same axes for focused needle plots (ie., x = proteins, y = pvalue) as the broader one in Fig 2a. Alternatively, consider moving 2A into the supplementary. Are any of these proteins from figure 2A downregulated?

Following your suggestion, we have made the necessary adjustments to ensure uniformity across the figures. Specifically, we have revised the focused needle plots in Fig. 2b to preserve consistent axes as those in Fig. 2a. We think that the Fig. 2a allows appreciating the comprehensiveness of our Complement proteome evaluation and we would like to keep it.

Great question. We have not identified downregulated proteins. Other study designs may be better powered to identify protective proteins.¹⁸⁻²⁰ Our study well powered to detect risk factors resemble other biomarker studies in the field, which mostly constitute risk factors. We added comments to the **updated Suppl. Table 11 and the Discussion**.

Figure 3: There seems to be a typo in the caption as it refers to “Figure 2a, c.” More explanation is required. This figure was confusing and hard to understand. What is included in M1?

We appreciate the comments and we see how this could have been confusing. Respectfully, *sensu stricto* this was not a typo, as Fig. 2a was the end of the sentence, whereas the “c,” describes the following panel of Fig. 3. Lower case “c” is used as per the journal's requirements for referencing sub-panels in figures. We have rephrased it and moved to a new paragraph to minimize the confusion for the reader. Thank you.

In response, we have **updated Fig. 3c** to improve its clarity and removed the term “M1” from the figure. We now explicitly state that it refers to a model adjusted by age and sex. The subsequent four models depicted in this figure represent partially adjusted models that evaluate potential confounding by key covariates. This plot offers a high throughput look on how P-values for all Complement protein associations with kidney failure risk change with each covariate adjustment.

Figure 4a: shows good agreement of protein risk between the 2 types of diabetes, in terms of significance. Why did the authors correlate p values and not the actual risk variable (are the magnitudes similar? Direction of risk the same?) It may flow better with the work (discovery to extension) in presenting Figure 5 before Figure 4.

Thank you for your suggestions. We are now presenting both p values, and hazard ratios from the model (**new in Fig. 4a**). In terms of the figure order, we can see it both ways. We, respectfully propose to keep the current order of Figures 4 and 5; the reason being that all the Figures 2-4 evaluate the urinary proteome so they present continuity, whereas Fig.5 expands to other matrices.

R2Q10. Writing. Certain words such as “complement,” “extension,” “discovery.”

“complement”. The noun “Complement” is intentionally used with a capital “C” throughout the manuscript when referring to proteins or transcripts of the Complement system or pathway, in order to distinguish it from the non-technical terms as the noun or the verb (“complement”, “to complement” or “to be complementary” meaning “to complete or enhance by providing something additional” as per Merriam-Webster dictionary). We hoped it would improve clarity for the readers. Pubmed and other engines do not distinguish technical from non-technical terms, making searches on the topic of the Complement system a bit more difficult. If the Reviewers think that this departs from the writing convention, we will be more than happy to remove the capitalization from the manuscript.

“extension”. We have thoroughly considered the following terms for our early-to-moderate DKD cohorts:

- *replication* (arguably, the fact that findings are present in advanced stages, but weaker in earlier disease, does not mean that they are not “replicated”; as it is a different disease phenotype);

- *validation* (arguably, weaker associations in earlier disease do not mean that the results in the advanced disease are not “valid” or “validated”);

- *extension* is a term sometimes used in clinical trials referring to extending the follow-up time.²¹ Here, we are technically evaluating whether our findings in advanced DKD “extend” to earlier stages of the disease.

“discovery”. Our two cohorts with advanced DKD may be considered a “discovery” as it all starts with high-throughput proteomics. Prioritizing signals concordant for type 1 and type 2 diabetes among these cohorts may be considered an equivalent of an internal validation, somewhat resembling a cross-validation. The findings are very strong, concordant between the two cohorts, significant after adjustment for multiple testing and after clinical covariates, validated across matrices, across molecule types and extended to earlier stages. In this context, the advanced DKD cohorts are way more than just a discovery. It is also worth mentioning that our study presents a translational project offering multiple lines of evidence, thus typical epidemiological conventions may not apply.

After having a closer look; and in order to reduce the potential confusion as raised by Reviewers 2 and 4, we **removed the terms of the discovery and extension**, as they may have not best reflected our study design (**multiple changes throughout the manuscript**). We added a few thoughts on our study design in the **discussion**. Please see also **R4Q1**.

The authors do not write out “DKD” in the first paragraph of the introduction.

We apologize for this omission. It has been corrected.

Discussion is quite long, with the first sentence being too wordy.

Discussion has been shortened.

Consider reworking the title, as “four cohorts” may be misleading.

Thank you for this valuable thought. We updated our title to:

Urinary Complement proteome strongly linked to diabetic kidney disease progression

Reviewer #3 (Remarks to the Author):

Zaipul I. Md Dom et al. carried out biostatistical analyses of urinary proteome in four prospective cohorts of 833 subjects with either type 1 or type 2 diabetes in an attempt to understand the progression of diabetic kidney disease and to obtain potential biomarkers. They obtained interesting findings that the most enriched pathways belonging to the Complement system. One strength of the study is that they use four 10-years follow-up cohorts of subjects with type 1 and type 2 diabetes. The other strength of the study is that they compared the results across three specimens, namely urine, plasma, and kidney tissues, to reveal the source of the biomarkers (Complement proteins). However, there are some concerns and unsolved issues as mentioned below.

Thank you very much for this comment.

Major points:

R3Q1 Table 1. The gender of the subjects is not balanced within the same group, namely within the advanced DKD (female : male = 44% : 56%) or within the early-to-moderate DKD (female : male = 30% : 70%), as well as between the two different groups.

It is such a valid point that **sex as a biological variable should always be given attention** and its importance has been well-recognized by the NIH and others.²² Our study invited all genders/sexes. The sex distribution of more prevalent male subjects in our study population reflects the natural history of the disease.²³ Moreover, the sex distribution is also quite similar to other recognized prospective observational or interventional studies of diabetic kidney disease in either diabetes type, as listed in the **new Table R1**.

Noteworthy, in our empirical data, **we did not observe interactions** between sex and any of the top Complement protein. We present Complement proteins by sex and prospective caseness status (**Ext. Data Fig.3**). Our multivariable analyses were also adjusted by sex, where we observed either minimal or no confounding.

Another important aspect of this debate is that **sex differences have also been recognized in the innate and adaptive immune responses at the molecular level**.²⁴ Sex differences in susceptibility to lupus, another kidney disease with a significant Complement engagement, may be linked to Complement gene/protein differences due to sex.²⁵ Epidemiological studies have also shown that more women are afflicted with lupus, another kidney disease with a significant Complement engagement.²⁶

We refrained from designing the study population balanced by sex because this would result in a smaller study population and fewer kidney outcomes compromising the study power. Further studies, specifically designed and powered may provide a final answer as to whether there is a sex difference relevant to the Complement engagement in diabetic kidney disease progression.

We have expanded comments based on the discussion above in the **Suppl. Table 11** and in the discussion.

R3Q2 2. Diabetes type is a concern. In the advanced DKD (type 1 : type 2 = 62% : 38%); in the early-to-moderate DKD (type 1 : type 2 = 39% : 62%). Since type 2 diabetes is usually insulin-resistant while type 1 is insulin-dependent, they have different latency (Table 1) in developing DKD and associate with different molecular pathogenesis. In the very beginning, if the authors separate the type 1 and type 2 patients and separately carry out the analyses, the results are expected to be different. For example, they may find different Complement protein and/or different ranking that associated with type 1 and type 2. They may also find features (markers) unique to type 1 and type 2, as well as features common to both type 1 and type 2. In the

present results of the manuscript, what the authors found are features (markers) common to both type 1 and 2 since they started with a collection combined both type 1 and 2 patients. Data adjusted for clinical covariates (e.g. sex, diabetes type) is a statistical method, which is different from an analysis using separate patient groups of type 1 and type 2 in the beginning.

We absolutely agree that the etiology, clinical manifestation and the temporal relationships of diabetic kidney disease between type 1 and type 2 diabetes are different. It is reflected in the length differences of diabetes duration of our study subjects between type 1 (above 30 years on average) and type 2 diabetes (15 or 11 years of diabetes on average). Our initial phase used a two-cohort approach to maximize the study power to identify molecular risk factors in subjects with advanced diabetic kidney disease. Our study may have potentially not identified diabetes type-specific signals. We comment on this in the study limitations in the Discussion and in the **Suppl. Table 11** on the representativeness of our study population. Please see also responses to **R2Q3**.

R3Q3. Minor points:

Figure 2a, the needle plot, on the x-axis, proteins are ordered by the pathway, the UniProt ID and the protein name. Some of the color between groups are too similar to tell the difference. In addition, one vertical needle represents P value of one Complement protein. The authors are suggested to indicate the name of each needle.

Thank you for your constructive feedback.

We have updated Fig. 2a by revising the color scheme for better visual distinction between different pathways. The new colors should provide a more noticeable contrast between groups.

Regarding your suggestion to link protein names on the x-axis, now in the Fig. 2 legend, we refer to the **Suppl. Table 4** that provides this information in a tabular format. We believe this approach is valuable because the table not only provides P-values linked to the figure but also includes effect estimates and several additional analyses. We were not successful in finding an optimal solution for labelling 110 proteins in the graphical format.

Also in response together to R4Q8, we have prepared **new Suppl. Table 10** listing all 110 proteins according to the corresponding pathway in the figure, which we believe provides comprehensive information for readers. Please kindly see also our responses to R2Q9.

Reviewer #4 (Remarks to the Author):

This manuscript by Dr. Niewczas and colleagues summarizes findings from an association study linking urine protein levels to incident adverse kidney outcomes (kidney failure, a composite endpoint of kidney failure and at least 40% loss of eGFR, and decline in eGFR) in four prospective studies of participants with type I and type II diabetes. The authors chose a discovery-validation design with each one type I and one type II diabetes collective, and quantified levels of ~1,300 urine proteins with Somascan aptamer technology. Urine protein levels were compared to their corresponding plasma and kidney tissue counterparts, as well as to classical molecular markers of kidney injury and diabetic kidney disease (DKD). The authors find that proteins belonging to the complement pathway are strongly enriched in persons reaching the kidney endpoint, and that high levels of these proteins in urine predict adverse kidney outcomes better and are linked to the kidney proteome much better than their plasma counterparts. The results suggest the importance of the complement system in the progression of DKD.

R4Q0. The manuscript is well written, and deals with a clearly important and increasingly common public health concern, DKD. The authors are to be commended on the comprehensiveness of their study design, including multi-matrix protein quantification, validation of aptamer quantification with orthogonal technologies, comparison of type I and type II DKD, and a comparison of classical biostatistical approaches to data analysis with newer machine learning methods. However, there are also limitations.

The major limitations of this study is the lack of an independent replication sample, in which to confirm associations and assess the predictive power of the urine protein biomarkers. The discovery results from this study are not independently validated. The study design is presented in a misleading manner (e.g., presenting this as a four cohort design, when in fact only selected discovery proteins were quantified in the extension with a different technology).

We very much appreciate this truly comprehensive review of our manuscript, careful considerations of its multiple components and the positive feedback. In response to the major critique, we are adding an independent replication sample to our study comprising Diabetes Heart Study participants. We have modified the description of our study design. We thank the Reviewer for these changes as they will benefit the reader. Detailed responses are below.

Comments about these points, as well as other limitations, are listed below, along with some suggestions:
Results:

R4Q1. Page 5, Figure 1: the authors state that the figure provides an overview of the study framework, but in fact the figure in its current state does not provide a very informative overview of the study design. The figure should include the sample sizes of each sample. Further, the figure needs to show which measurements and which analyses are performed in both discovery and extension, and which only in the discovery cohort. It is important that readers are informed whether and which findings were independently assessed for validation in the extension study.

We have dedicated substantial effort to clarifying our study design in response to the critiques, **R4Q1-4** and **R2Q10**. As a result, the abstract, the manuscript text and the **updated Figure 1** has been substantially revised. We are also changing our **title** to “Urinary Complement proteome strongly linked to diabetic kidney disease progression”, so that it better reflects our study design.

We have thoroughly considered the terms designing our advanced and early-to-moderate DKD cohorts.

Discovery cohorts. In our humble opinion, our two cohorts with advanced DKD may be considered a “discovery” as it all starts with high-throughput proteomics. Prioritizing signals concordant for type 1 and type 2 diabetes among these cohorts may be considered an equivalent of an internal validation, somewhat resembling a cross-validation. The findings are very strong and validated in many different ways: i) they are concordant between the two cohorts, significant after adjustment for multiple testing and after clinical

covariates, ii) validated across matrices, iii) and across molecule types (RNA and protein) and extended further to earlier stages. In this context, the advanced DKD cohorts may be considered more than just a discovery.

Extension cohorts. When it comes to our early-to-moderate DKD cohorts:

- *replication* (arguably, the fact that findings are present in advanced stages, but weaker in earlier disease, does not mean that they are not “replicated”; it is a different disease phenotype);
- *validation* (arguably, weaker associations in earlier disease do not imply that the results in the advanced disease are not “valid” or “validated.” Noteworthy, we offer multiple lines of validation in our manuscript such as: assay validation, kidney tissue validation at the protein and RNA level);
- *extension* is a term sometimes used in clinical trials referring to extending the follow-up time.²¹ Here, we are technically evaluating whether our findings in advanced DKD “extend” to earlier stages of the disease.

It is also worth mentioning that our study presents a translational project offering multiple lines of evidence, thus typical epidemiological conventions may not apply. To reduce a potential confusion as raised by the Reviewers 2 and 4, we are **removing the terms of the discovery and extension**. Please see also **R2Q10**.

R4Q2. On page 9, lines 170 ff: please clearly state if plasma and kidney tissue proteins were quantified in all participants of the discovery cohorts. This can be included as sample size information in the revised figure 1, it should not just be hidden in a supplementary table.

We have added this information, respectively to the text, to the **updated Figure 1** and to the **updated Fig. 5** legend, respectively. Please kindly also see responses to R4Q1.

R4Q3. Each main figure should contain information in the figure panels (e.g., as part of the title or axis labeling) whether it is based on the discovery study only. It is very misleading if the authors present this as a four cohort validation – extension study design, when in fact only secondary analyses included information from the extension study, and only for six out of the 1300 proteins.

We have added explicit information on the study groups to the Figure panels or titles (**updated Figs. 2, 3 and 5**). Please kindly see also responses to R4Q1.

R4Q4. Still along the same lines, only Figure 4 b and c seems to contain data from the extension cohort. Only at the very end of the manuscript (page 30, Methods), did I find information that in fact the extension cohort only performed targeted measurements of complement proteins and not across the urine proteome. This does not become clear in the text. Figure 1, abstract and manuscript need to be rephrased as a two-study discovery cohort only design. The two-study extension is only a secondary analysis and should therefore not be shown on the same level as the discovery.

Please see responses to the critique, R4Q1.

R4Q5. The plots in Fig 4b and c show that the associations with annual eGFR slope and with the composite kidney outcome are clearly attenuated in comparison to the discovery cohort. I could not find easily if these results are from the base or the adjusted model. They should show results from the adjusted model. Please clarify and include adjusted results, if not adjusted already.

Thank you for this comment. Plots in **updated Fig. 4b** show effect estimates across five cohorts (in the former four cohorts **plus in a new cohort**) for the continuous outcome (GFR slope) maximizing the study power. Plots in **updated Fig. 4c and d** (formerly 4b and c) show crude, but now also adjusted effect estimates for the advanced and early-to-moderate DKD cohorts for the intermediate kidney outcomes (GFR slope and the composite, binary outcome). Corresponding **new Suppl. Table 6a-f** present effect estimates, confidence intervals and strengths of the association (p values).

Our advanced DKD cohorts have had long follow-up and the incident kidney failure was frequent. Therefore, we have comprehensively adjusted our Cox model by the clinical covariates (age, sex, race, diabetes type and duration, body-mass index, systolic and diastolic blood pressure, hemoglobin A1c (HbA1c), GFR, albuminuria, cholesterol, smoking status, insulin use, renoprotective/other antihypertensive, and lipid-lowering treatments), shown in Fig.3a.

Our early-to-moderate DKD cohorts had shorter follow-up and kidney outcomes were less frequent. Due to a limited power, we were able to adjust our models for fewer covariates (age, sex, HbA1c GFR and diabetes type). We did not adjust these models for albuminuria, as it may have led to overadjustment. Arguably, albuminuria may likely be a part of the causal pathway together with Complement. Our main goal was to appreciate the etiology of the disease, rather than develop a prognostic model, similar to modelling approaches described before.^{8,27} For the Reviewer's reference the albuminuria-adjusted models are enclosed (**new Table R2**).

R4Q6. The authors interpret the attenuated associations in the extension in Figures 4b and 4c to mean that associations in advance kidney disease are stronger than in early-to-moderate DKD, but in fact it may just be winner's curse or related to the different assays that was used in discovery and extension.

In support of winner's curse, it is quite unusual that the prediction of outcomes at 10 years (i.e, more distantly in the future) performs better than at 3 or 5 years, as shown on page 10, 198 ff. How do the authors explain this?

We absolutely agree. We cannot entirely exclude the possibility of winner's curse. We provide here a number of arguments, which we believe make this potential phenomenon a less likely explanation of our findings.

Analytical considerations.

In order to address a fair concern of using two different assays in the advanced and early-to-moderate DKD, we now provide **new empirical data in the form of targeted measurements** for two out of the top 5% proteins in the advanced DKD cohorts (165 subjects with T1D and 107 subjects with T2D, respectively; 89% of the study group due to availability) performed with the same assays as the one used in the early DKD. Sample volumes are limited in the precious collections of the Joslin Kidney Study, therefore two out of 6 proteins were measured. Patterns of the effect sizes with targeted measurements remarkably resembled those performed with aptamer proteomics. The forest plot illustrating effect sizes by either method across 5 cohorts is now provided as a **new Suppl. Fig. 3** with a corresponding method description and a short note in Discussion. Thank you for inspiring us to offer this valuable addition.

Biostatistical considerations

As per Ioanidis,²⁸ "flexible analyses coupled with selective reporting may inflate the published discovered effects." To address this possible component of the "winner's curse", we refined our binary outcome. Previously, we presented 40% GFR loss for the advanced DKD and 30% GFR loss for early-to-moderate DKD cohorts. We now report 30% GFR loss or kidney failure across all cohorts to offer more fair comparisons. Please note that our results are reproducible across 2-3 kidney outcomes reported in the study (GFR slope, GFR loss and incident kidney failure (advanced DKD only)), **updated Fig.4d, new Suppl. Table 6**.

Moreover, to evaluate the potential issue of an unmeasured confounding, we added E-value for the top Complement associations (30% GFR loss and/or kidney failure); **new Table S6f**. E-value is a parameter displaying the minimum strength of association that an unmeasured confounding factor would need to have with both the exposure and the outcome to explain away an exposure-outcome association of interest.²⁹

It is worth mentioning that the Joslin Kidney Study cohorts have provided highly reproducible insights into the biomarkers and disease processes over the years. For example, Joslin Kidney Study was the very first to demonstrate that TNFR is an extremely robust prognostic biomarker of diabetic kidney disease progression.^{30,31} This phenomenon has now been replicated in multiple cohort studies. Notably, the replications were reported with targeted³²⁻³⁴ and high-throughput assays^{8,10,11}, similarly to our study. As per the meta-

analysis by *Liu et al*³⁵, strong associations between circulating TNFR and kidney outcomes were reported in 31 studies, replicated in 30 of them and featured the largest effect sizes among targeted biomarker studied in circulation.

Indeed, the strengths of the associations for the 10-year outcomes are more pronounced than the models for the shorter follow-up, likely due to the following factors.

The data used to fit the model with length of observation of 10 years contain more information (due to less censoring). Noteworthy, there have been more outcomes for the longest follow-up than for the shorter follow-up times, resulting in narrower confidence intervals for HR compared to models with 5 and 3 years length of observation. We have now added the person-years, and outcome frequencies (**updated Suppl. Table 9**) for easier appreciation of our results. We have also added information on the proportionality testing that implies that the effects of the variable are constant over time at the hazard rate.

R4Q7. Page 6, lines 118 ff: please provide the adjusted results that correspond to the unadjusted results in lines 101 ff.

It has now been provided.

R4Q8. In order to interpret the enrichment in complement markers in the discovery better, it is important to know their correlation. Please include a correlation matrix between the 66 significant proteins from the discovery cohort in a supplementary table and visualize as a correlation matrix sorted by pathway. Please provide such plots stratified by albuminuria category (A2, A3) at baseline.

Thank you for this comment. We very much agree that the evaluation of correlations among proteins is important for multiple reasons. This comment inspired us to provide a correlation matrix among all 110 Complement proteins measured. One can appreciate interesting, stronger correlations among Complement factors of the alternative pathway or among the coagulation factors or weak correlations between C7 and other proteins (**new Suppl. Table 11**).

Following the Reviewer's request, we also provide correlation matrices stratified further by the albuminuria status. Correlation patterns seem similar (**new Table R3**).

Please note that we do provide two approaches demonstrating enrichment in Complement (pathway-driven, when a pathway is evaluated one at a time, and data-driven, when all Complement pathways are combined to one category, Ext Data Fig. 1). Pathway annotation comes with certain limitations. Our careful curation of the Complement protein annotations was an undertaking that consumed several weeks. Respectfully, a quality curation of the pathway annotations of the entire proteomics array and the related matrix seem to us beyond the scope of this project. These important questions will be definitely pursued in further studies.

R4Q9. Page 9, lines 185 ff: in this Reviewer's opinion, the single cell data don't add important information to the manuscript and can be removed. The authors state themselves in the introduction that the transcriptome and the proteome are poorly correlated.

We appreciate the suggestion to decrease the complexity of our paper. However, we feel that the kidney tissue transcriptomics and proteomics data are its important parts. They are novel as they demonstrate that select proteins are synthesized in the kidney and increased in subjects with DKD. They bring focus to the local kidney production, by contrast to many other Complement-mediated kidney diseases where Complement dysfunction is systemic. Those data seem further to be of particular interest to other Reviewers. As per Reviewer #3 "The other strength of the study is that they compared the results across three specimens, namely urine, plasma, and kidney tissues, to reveal the source of the biomarkers (Complement proteins).", whereas Reviewer #1 states that "Kidney tissue transcriptomic data showed a strong concordance with urinary findings." and focuses

his/her review on the kidney tissue data. These data help us build a case on the protein source being likely production, rather than a simple effect of filtration (questions of Reviewers #1 and #2).

R4Q10. Page 10, lines 203 ff: the results of the clustering analyses are driven by the fact that they are based on proteins that are associated with outcomes in the same sample. The prognostic power (composite endpoint) should be assessed in the extension study, for the markers available there.

We are grateful to the Reviewer for this great idea. We gladly offer the external validation in the Extension in addition to the internal validation in the Discovery cohorts. As shown in the **new Ext. Data Fig.7a-d**, clusters informed by top proteins measured in early-to-moderate DKD cohorts significantly differentiate risks of prospective kidney outcomes.

R4Q11. Discussion: the discussion of the findings is long, but the lack of independent replication, a major limitation, is not adequately addressed. Many of the details can be shortened, as the findings from this first urine proteomics study need to be validated in an independent replication sample first.

We have shortened the content of the original discussion, but we also modified it to address all the excellent suggestions offered by the reviewers.

We now provide an independent replication cohort of Diabetes Heart Study. Please see responses to R4Q0.

Minor comments:

R4Q12. In Table 1, please include information about race.

It has now been added.

R4Q13. Page 6, line 107: it seems unlikely that only 14 persons died over the course of follow-up (10 years). Please double check.

We very much appreciate the reviewer's careful reading of our manuscript. Please note, we have collected data on neither post-kidney failure mortality nor on non-fatal CVD events, which likely would have been much higher. We now discuss this as a limitation of our study in the **Discussion**.

We would like to emphasize that our mortality data were based on queries against the National Death Index, matched by a unique identifier, so they should offer a high level of accuracy. Yes, we did double check our records. In order to offer further reassurance, we have now reviewed an additional query against the NDI from 2017, which was performed 4 years after the outcomes were locked in for the current study. The additional follow-up would result in 2 more cases of death for subjects with T1D and 11 more cases of death for subjects with T2D.

In our study, subjects with kidney failure as a primary cause of death have been classified as kidney failure cases (KF Died, n=9) together with kidney failure cases in subjects who remained alive (KF Alive, n=117). We apologize for this omission; the information has been added to the **Method section**. Moreover, for the Reviewers' information we provide the boxplots of top 5% Complement proteins split by these subgroups (**new Fig. R2**). Relevantly, Complement proteins were comparable in both subgroups of cases with the kidney failure (in among those who remained alive as well as among those who died with kidney failure), and noticeably higher than the remaining groups, alive subjects without kidney failure or subjects who died due to other causes.

Furthermore, the relatively low mortality risks in the Joslin Kidney Study populations in comparison with other cohorts have been well-documented (*Skupien and others in Diab Care 2019*)³⁶. This international, prospective study comprised over 15 thousand person-years and consisted of the following cohorts: Joslin Kidney Study,

FinnDiane, Steno Diabetes Center and INSERM. From among subjects with T1D, A3 albuminuria and G3 kidney function, the incidence risks of kidney failure were 53.7 per 1,000 patient-years, whereas the mortality rates were over six times lower (8.1 cases per 1,000 patient-years). By contrast, although the kidney failure rates were similar in Steno and INSERM cohorts to those at Joslin (53.4 and 45.2, respectively), the mortality rates were over two times more frequent (23.3 and 21.0, respectively). We have now commented on this phenomenon in the **updated Suppl. Table 11** on the study generalizability.

R4Q14. Page 11: the results end quite abruptly, consider including one summarizing sentence.

We have added a summarizing sentence. Thank you.

R4Q15. Figure 4 a: putting the base and adjusted estimates side by side would be better to make apparent if their confidence intervals overlap.

We are now providing adjusted estimates in the **updated Fig.3, the Fig.4c-d, and new Table R2.**

Tables and Figures for Reviewers only:

Table R1 (new). Sex distribution in select, recognized prospective study populations with similar diabetes and kidney phenotypes to our study populations.

Study name	Study design	Diabetes type	Kidney disease stage	Total number of subjects	Female (%)
Joslin Kidney Study	Observational	Type 1	Moderate-to-advanced	432	42.8
FinnDiane cohort	Observational	Type 1	Moderate-to-advanced	486	39.7
Steno cohort	Observational	Type 1	Moderate-to-advanced	368	39.9
INSERM	Observational	Type 1	Moderate-to-advanced	232	40.1
PERL	Interventional (allopurinol)	Type 1	Early-to-moderate	530	33.8
DAPA-CKD	Interventional (dapagliflozin)	Type 2 and non-diabetic kidney disease	Moderate-to-advanced	4304	33.1
FLOW	Interventional (semaglutide)	Type 2	Moderate-to-advanced	3533	30.3

Primary reports on the listed studies were reported elsewhere.³⁶⁻³⁹

Table R2 (new). Top Urinary Complement Proteins and Kidney Outcomes in the Advanced and Early/Moderate DKD in the albuminuria-adjusted models.

Please refer to the separate excel file.

Table R3 (new). Relationships among urinary Complement proteins, sorted by pathway, and stratified by albuminuria category, in the advanced DKD two-cohort study.

Please refer to the separate excel file.

Fig. R1 (new). Gene expressions of CD55 and CD59 in diabetic vs control kidney tissue. Data are from our study are shown as bubble plots in all kidney tissue examined by caseness comparing diabetic kidney tissue (red) vs control tissue (blue). The DAF protein is a product of the CD55 gene. Dot size in both panels represents the percent of cells expressing the gene of interest, whereas the color intensity corresponds to the expression.

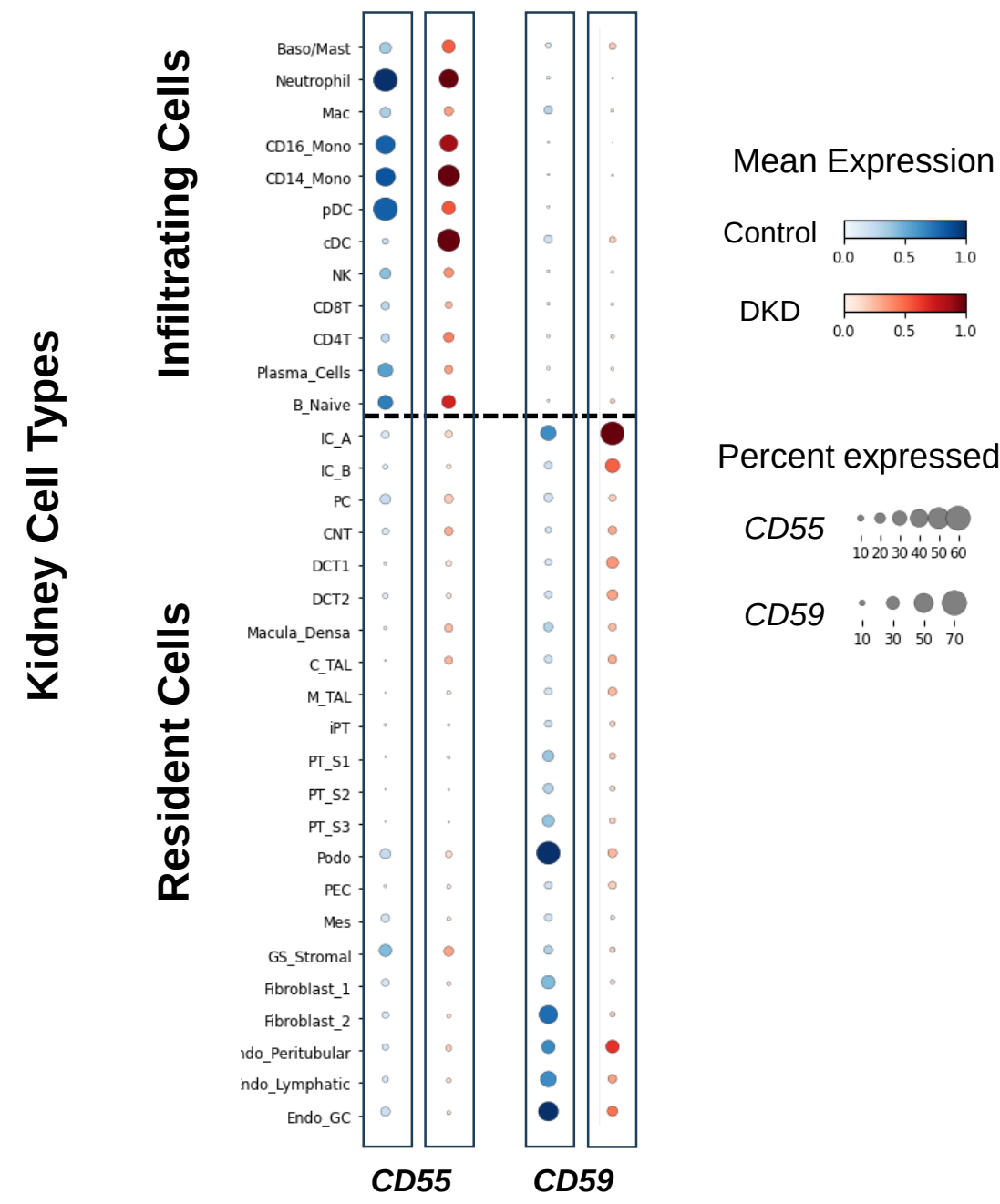
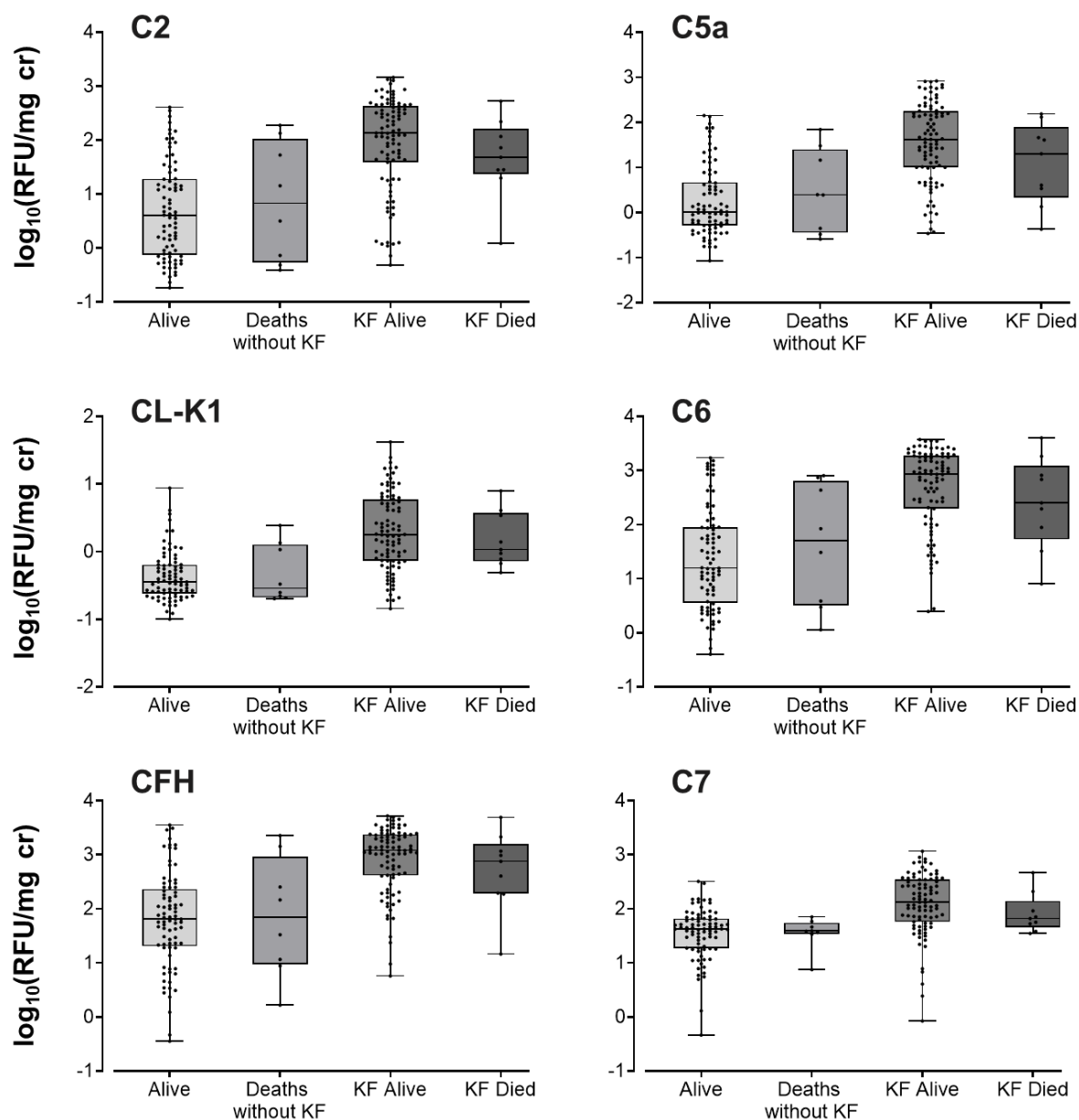


Fig. R2 (new). Boxplots show the relative concentrations of top 5% Complement proteins in urine in subjects with T1D from the advanced DKD cohort, who remained alive without kidney failure (n=81), subjects who died unrelated to kidney failure (n=8), and subjects who developed kidney failure and remained alive (n=91) or died (n=9).



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Responses to Reviewers

We very much appreciate the positive responses of the reviewers (“authors have put in tremendous work to fulfill the diversity aspect...” as an example). We are glad that Reviewers 1, 2 and 3 feel that we have adequately addressed their critiques. Below, we also provide a more detailed response to the remaining critique of Reviewer #4.

REV4: “The authors have thoroughly revised the manuscript, and added an external validation sample, as requested. I note that the effect estimates with respect to slope and composite kidney endpoint in this new external sample, while concordant in direction, are smaller than in the other two early DKD studies for the tested top proteins and also smaller than in advanced DKD. Several of them are not even statistically significantly associated with adverse kidney events in this new sample (see Fig. 4b). Moreover, the three early DKD validation cohorts do not adjust their Cox models for albuminuria. Hazard ratios in 4a still seem to be presented unadjusted, which may be misleading. I suggest updating 4a to show adjusted estimates to be concordant with the other panels, and to adjust the early DKD cohorts for UACR as well, even if not all cohorts are feasible. This reviewer believes it is important to given a realistic depiction of how much these urinary proteins can add above and beyond UACR. Other than this point, the authors have adequately responded to my comments, and their efforts are very much appreciated.”

Answer to REV4: We thank the Reviewer for this comment.

We now provide associations of top Complement proteins adjusted by albuminuria as a part of Supplementary table 6.

We also find it remarkable that the effect estimates are concordant across 3 cohorts of differing diabetes type, calendar years and site of recruitments and racial background. We agree that the Collectin Kidney 1 (CL-K1) is not replicated in early DKD cohorts, and it is acknowledged and discussed in the manuscript. We also acknowledge that the confidence intervals for C5a and CFH cross the x axis at 0 for one of the five cohorts (early/moderate DKD in T2D-AA), as reflected in a new sentence we have added to the manuscript. Due to the paucity of kidney failure in early/moderate DKD (as discussed in the manuscript and presented in the Supplementary Table 1), we do not have sufficient power to create Cox models in these cohorts. Therefore, we are unable to perform additional analyses as suggested by the Rev4. Kidney outcomes are listed in great detail in Supplementary Table 1 and analyses for the continuous GFR slope and binary outcome of 30% GFR loss/kidney failure are shown in Figure 6 and Supplementary Table 6.